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Relationships of the Predisposition to Convulsions with the Action Potentials of the Autonomic Nerves and the Brain

I. Effects of Several Autonomic Drugs on the Predisposition to Convulsions, the Action Potentials of the Autonomic Nerves and the Brain

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Since the conception of the predisposition to convulsions was first introduced by Förster,[1] many studies on this problem have been published. Information as to the relationships between autonomic nerves and convulsions is still insufficient though several reports are available such as those by Kessler & Gellhorn,[2] Yakovlev,[3] Trinker & Serota,[4] Gellhorn and others,[5] Horiguchi and Takeya,[6] and Kanazawa.[7),8] Concerning the action potentials of peripheral nerves, on the other hand, several reports have appeared following the brilliant work by Erlanger & Gasser.[9] Recently, Sakamoto[10] and Tanaka[11] have published studies on the action potentials of autonomic nerves, in which they deal only with the spike potential like many other workers, however, Orihara[12] in our clinic has made comprehensive studies of the action potentials of autonomic nerves and the brain, in which potential changes were recorded by means of an electroencephalograph and analysis was made of the period and amplitude of the curves thus obtained. Hereupon, I have undertaken a series of studies of to elucidate this problem of the predisposition to convulsions with special reference to the action potentials of autonomic nerves and the brain. The present paper is the first report of these studies, in which five representative "autonomic drugs" were used.

Experimental

Methods

The experimental animals used were 25 unanesthetized adult rabbits weighing about 2 kg. Both the sympathetic and the parasympathetic (vagus) were carefully separated 3 cm. or longer from the surrounding

tissure after a median incision was made at the neck of a rabbit fixed on its back. The separated portion of each nerve was lightly lifted by a pair of hockshaped electrodes with their tips held 1.5 cm. apart and covered with wool fibers moistened with 0.09 per cent saline solution (Fig. 1). The electroencephalogram (EEG), the electrocardiogram (ECG), and the respiration curve were simultaneously recorded by Berger's method of fronto-occipital induction,[13] Wilson's V_4 method of thoracic wall single electrode induction,[14] and the zink-zink sulfate method, respectively.

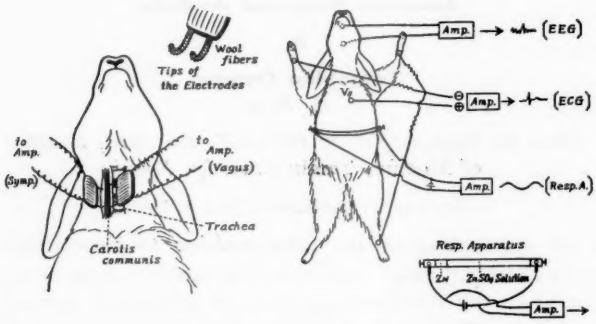

Fig. 1. Diagrams showing the methods for recording the action potentials of the autonomic nerves (left), and for obtaining the EEG, ECG, and respiration curve (right).

The changes in action potential of the autonomic nerves and the brain were analyzed by means of the frequency histogram after Motokawa,[51] which was constructed with the logarithm of the frequency of waves as abscissa and the rate of occurence of waves belonging to each group of frequency as ordinate. Being obtained with a single electrode, the ECG was used only for calculating the pulse rate, which served, together with the respiration curve, as an aid for discerning the disorder of the autonomic nerves. The amplifier used was an 8-channel ink-writing electroencephalograph (Sanei Measuring Apparatus Co.), and the characteristic frequency of the entire system was 0.5 to 42 cycles per second.

For measuring the predisposition to convulsions, the metrazol method was used: A rabbit was injected with 0.5 per cent metrazol through an ear vein at a constant rate of 0.1 cc. per 30 seconds; from the total amount of metrazol required for the start of tonic convulsions "convulsive threshold of metrazol" was determined; the threshold per kilogram body weight was then calculated, and its reciprocal multiplied by 100 was used as index of the predisposition to convulsions (hereafter to be referred to as "PTC"). The PTC measurement was performed soon after the

recording of each action potential was finished.

The following five representative so-called " autonomic drugs " were used: adrenaline (sympathetic stimulant), pilocarpine (parasympathetic stimulant), atropine (parasympathetic inhibitor), and hexamethonium bromide and chlorpromazine (sympathetic and parasympathetic inhibitor). Five rabbits were used for each drug; a solution of the drug was injected slowly into an ear vein in all the cases.

Results

Predisposition to convulsions of normal rabbits (Tables I, II)

The convulsive threshold of metrazol in a normal rabbit was 0.8 to 1.9 cc. (1.3 cc. on the average with 25 rabbits), i.e. 0.45 to 1.0 cc. per kg. body weight (average: 0.69 cc.); the PTC value was 100 to 225 -average: 152, thus showing a relatively wide variation from individual to individual. But the threshold variation in the same individual was only slight within a short period of time, being less than 0.03 cc. when the threshold per kg. body weight was measured twice at an interval of 7 to 10 days (the PTC difference being 11 at most).

The action potentials of the autonomic nerves and the brain, and the rates of

TABLE I

Predisposition to Convulsion by Metrazol of Normal Rabbits

No.	Weight (kg.)	Convulsive threshold (cc)	per kg.	PTC
1	1.80	1.20	0.67	150
2	1.40	1.40	1.00	100
3	1.90	1.25	0.66	152
4	1.70	1.45	0.85	117
5	1.70	1.60	0.94	106
6	1.80	1.30	0.72	138
7	1.40	1.30	0.93	108
8	1.80	1.25	0.70	144
9	1.60	1.45	0.91	110
10	1.70	1.60	0.94	106
11	1.60	0.80	0.50	200
12	1.70	1.25	0.74	136
13	2.20	1.45	0.66	152
14	2.20	1.20	0.54	183
15	2.80	1.40	0.50	200
16	1.70	1.25	0.73	136
17	2.20	1.45	0.66	151
18	2.80	1.90	0.68	147
19	1.80	0.80	0.45	225
20	2.20	1.20	0.55	183
21	1.80	0.90	0.50	200
22	1.70	1.30	0.77	131
23	2.20	1.40	0.64	157
24	2.80	1.40	0.50	200
25	1.80	1.00	0.56	180
Mean	1.92	1.30	0.69	152

TABLE II

PTC Change of the Same Normal Rabbits

No	Weight	Convulsive threshold by metrazol (cc)	per kg	PTC	Difference
1	1.8	1.20	0.67	150	0
	1.8	1.20	0.67	150	
6	1.8	1.30	0.72	138	− 3
	1.7	1.25	0.73	135	
11	1.6	0.80	0.50	200	−11
	1.7	0.90	0.53	189	
16	1.7	1.25	0.73	136	+ 6
	1.7	1.20	0.71	142	
20	2.2	1.20	0.55	183	− 8
	2.1	1.20	0.57	175	

pulse and respiration under normal conditions (Fig. 2, Table III)

The amplitude of the vagus action potential was 20 to 30 μV, and the peak of the frequency histogram lay between 39.2 and 24 cycles per second. In the sympathetic, the amplitude was a little larger (30 to 50 μV), but the peak of the histogram lay in the same range as in the vagus. In the EEG, the amplitude was about 50 μV, and the peak was situated between 39.2 and 24 cycles per second as in the cases of the autonomic nerves. The rates of pulse and respiration as examined with 10 rabbits were 218 to 300 (average: 264.7) and 30 to 45 (average: 38.9) per minute, respectively.

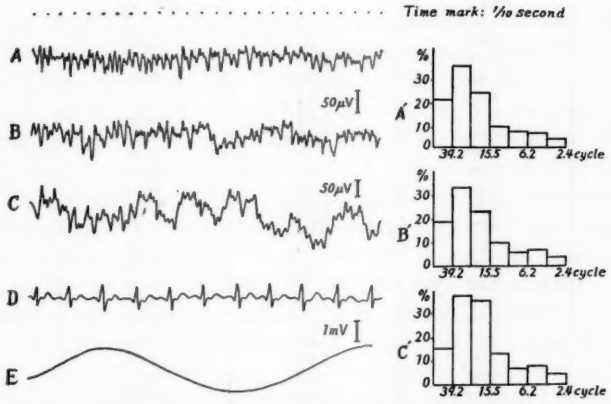

Fig. 2. Spontaneous action potentials and their frequency histogram of a normal rabbit. *A*, vagus action potential; *B*, sympathetic action potential; *C*, EEG; *D*, ECG; *E*, respiration; *A'*, *B'*, *C'*, frequency histograms for A, B, C. The explanation of the lettering and other expressions given above is common to all figures 2, 5, 7, 9, and 11.

TABLE III

Rates of Pulse of Respiration of Normal Rabbits

No.	Pulsations	Respirations
2	258.0	36.0
4	290.0	38.0
6	218.0	40.0
7	253.0	37.0
12	249.0	40.0
13	265.0	41.0
17	281.0	40.0
19	293.0	45.0
21	240.0	30.0
23	200.0	42.0
Mean	264.7	38.9

Effects of adrenalin on the PTC and the action potentials (Figs. 3, 4: Table IV)

A rabbit was injected slowly with 0.1 mg. of 0.01 per cent adrenalin hydrochloride per kg. body weight. Several minutes after this, a decrease of sympathetic fast waves (39.2 to 24 cycles per second) was observed in

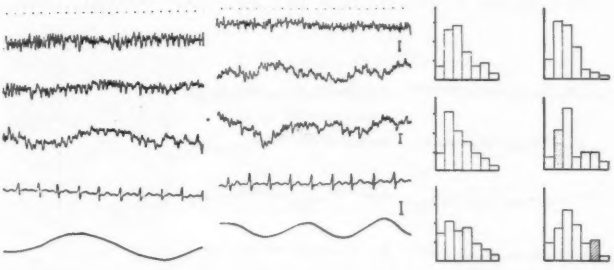

Fig. 3. Effects of adrenalin on action potentials, etc. Left to right: Action potentials before and after injection, and frequency histogram before and after injection. In the histograms, shaded column: Increasing change; dotted column: decreasing change. Lettering and other expressions as in Fig. 2.

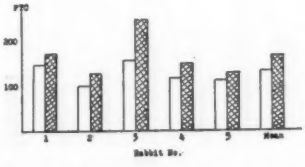

Fig. 4. Effects of adrenalin on PTC. White column: before injection, shaded column: after injection.

TABLE IV

Effect of Adrenalin on PTC

No.	Weight (kg.)	Before injection Threshold (cc)	per kg	PTC	After injection Threshold (cc)	per kg	PTC
1	1.8	1.20	0.67	150	1.1	0.61	163
2	1.4	1.40	1.00	100	1.0	0.71	140
3	1.9	1.25	0.66	152	0.8	0.42	238
4	1.7	1.45	0.85	117	1.2	0.71	142
5	1.7	1.60	0.94	106	1.5	0.88	113
Mean	1.7	1.38	0.82	125	1.1	0.55	159

all cases, though the amplitude showed no marked change; in the vagus, there were seen no noticeable changes. In the EEG, an increase of slow waves (6.2 to 3.9 cycles per second) was observed in 4 cases. The pulse rate did not change remarkably, while the respiration rate was increased. The PTC showed an increase in all cases.

Effects of pilocarpine on the PTC and the action potentials (Figs. 5, 6; Table V)

Several minutes after a slow intravenous injection of 1 mg. of 0.2 per cent pilocarpine hydrochloride per kg. body weight, there appeared

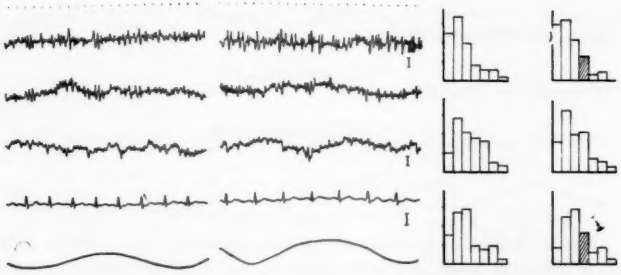

Fig. 5. Effects of pilocarpine on action potentials, etc. Left to right: action potentials before and after injection, and histograms before and after injection. Shaded column: increasing change. Lettering and other expressions as in Fig. 2.

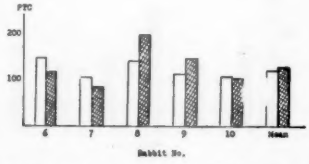

Fig. 6. Effect of pilocarpine on PTC. White column: before injection, shaded column: after injection.

TABLE V

Effect of Pilocarpine on PTC

No.	Weight (kg)	Before injection Threshold (cc)	per kg	PTC	After injection Threshold (cc)	per kg	PTC
6	1.8	1.30	0.72	138	1.55	0.86	116.0
7	1.4	1.30	0.93	108	1.70	1.21	82.6
8	1.8	1.25	0.70	144	0.90	0.50	200.0
9	1.6	1.45	0.91	110	1.10	0.69	145.0
10	1.7	1.60	0.94	106	1.65	0.97	103.0
Mean	1.7	1.38	0.83	121	1.38	0.84	129.0

in the vagus an increase of intermediate waves (15.5 to 9.8 cycles per second) in all cases and a slight increase in amplitude; the sympathetic was little affected. In the EEG, an increase of intermediate waves (15.5 to 9.8 cycles per second) took place in 4 cases. The rates of both pulse and respiration were decreased. The PTC was increased in 2 cases but decreased in the remaining 3 cases, the average value being a little higher than the normal.

Effects of atropine on the PTC and the action potentials (Figs. 7, 8; Table VI)

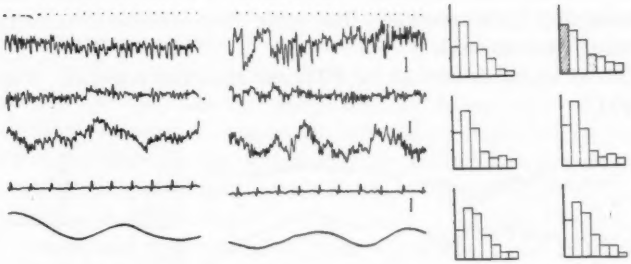

Fig. 7. Effects of atropine on action potentials, etc. Left to right, action potentials before and after injection, and histograms before and after injection, shaded column: increasing change, dotted column: decreasing change. Lettering and other expressions as in Fig. 2.

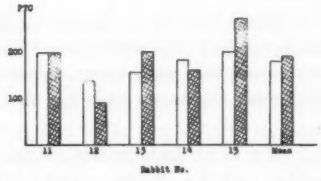

Fig. 8. Effect of atropine on PTC. White column: before injection, shaded column: after injection.

TABLE VI

Effect of Atropine on PTC

No.	Weight (kg)	Before injection Threshold (cc)	per kg	PTC	After injection Threshold (cc)	per kg	PTC
11	1.6	0.80	0.50	200	0.80	0.50	200
12	1.7	1.25	0.74	136	1.90	1.12	90
13	2.2	1.45	0.66	152	1.10	0.50	200
14	2.2	1.20	0.55	183	1.50	0.68	161
15	2.8	1.40	0.50	200	1.00	0.36	277
Mean	2.1	1.22	0.58	174	1.26	0.63	186

Several minutes after a slow intravenous injection of 0.1 mg. of 0.02 per cent atropine sulfate per kg. body weight, the amplitude of the vagus potential showed a remarkable increase in 3 cases, but became smaller than the normal in 7 to 10 minutes; there appeared an increase of vagus fast waves (39.2 cycles or more per second) in all cases and a decrease of intermediate waves (15.5 to 9.8 cycles per second) in 4 cases. The sympathetic and brain potentials showed no definite tendencies in their changes though affected to some extent. The pulse rate was increased a little, but the respiration rate was lower than the normal in 4 cases. The PTC was increased in 2 cases, decreased in 2 but remained unchanged in the remaining 1, the average value being about the same as (very slightly higher than) the normal.

Effects of hexamethonium on the PTC and the action potentials (Figs. 9, 10; Table VII)

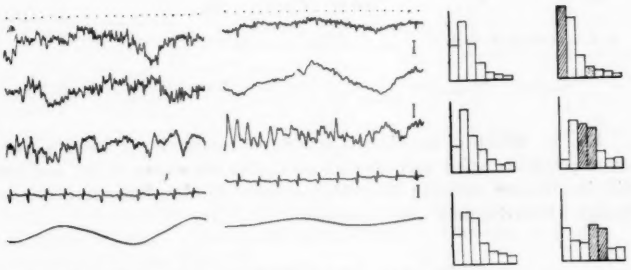

Fig. 9. Effect of hexamethonium on action potentials, etc. Left to right, action potential before and after injection and histograms before and after injection, shaded column : increasing change, dotted column : decreasing change. Lettering and other expressions as in Fig. 2.

When 3 mg. of 0.5 per cent hexamethonium bromide was injected intravenously into a rabbit per kg. body weight, the amplitudes of both nerves (especially the sympathetic) were markedly decreased after several

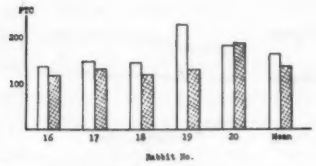

Fig. 10. Effect of hexamethonium on PTC. White colum : before injection, shaded column : after injection.

TABLE VII

Effect of Hexamethonium on PTC

No.	Weight (kg)	Before injection Threshold (cc)	per kg	PTC	After injectiou Threshold (cc)	per kg	PTC
16	1.7	1.25	0.74	136	1.45	0.86	117
17	2.2	1.45	0.66	151	1.65	0.75	133
18	2.8	1.90	0.68	147	2.40	0.86	117
19	1.8	0.80	0.45	225	1.40	0.78	129
20	2.2	1.20	0.55	183	1.15	0.52	191
Mean	2.1	1.32	0.61	168	1.61	0.75	137

minutes. In the vagus, there was seen an increase of fast waves (39.2 cycles or more per second) in all cases and a decrease of intermediate waves (15.5 to 9.8 cycles per second) in 4 cases. In the sympathetic, the amplitude was decreased so much that the frequency was measurable only in 3 cases, in each of which an increase of intermediate waves (24 to 9.8 cycles per second) was observed. In the EEG, intermediate waves (15.5 to 6.2 cycles per second) were increased pronouncedly. The rates of both pulse and respiration were decreased. The PTC showed a decrease in 4 cases and slight increase in the remaining 1 case ; this increase, however, fell within the range of normal variation in the same individual rabbit.

Effects of chlorpromazine on the PTC and the action potentials (Figs. 11, 12 ; Table VIII)

When 2 mg. of 0.25 per cent chlorpromazine per kg. body weight was injected, the action potentials of both autonomic nerves showed decreases in amplitude after several minutes, the change being very pronounced in the sympathetic ; there increased rapid waves in both nerves ; in addition, an increase of vagus intermediate waves (24 to 15.5 cycles per second) was seen in 4 cases. In the EEG, the period was prolonged in all cases, and slow waves (9.8 to 2.4 cycles per second) increased markedly. The rates of pulse and respiration decreased in all cases. The PTC showed a decrease in 4 cases ; though a slight increase was seen in the

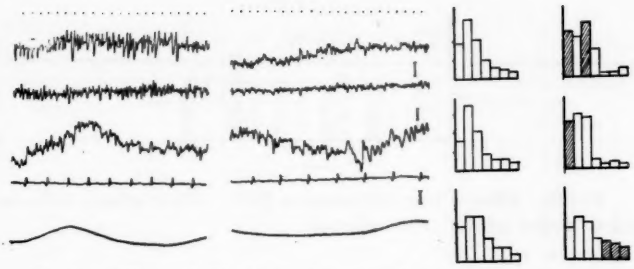

Fig. 11. Effects of chlorpromazine on action potential, etc. Left to right, action potentials before and after injection and histograms before and after injection, shaded column: increasing change. Lettering and other expressions as in Fig. 2.

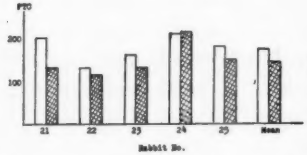

Fig. 12. Effect of chlorpromazine on PTC. White column: before injection. Shaded column: after injection.

TABLE VIII

Effect of Chlorpromazine on PTC

No.	Weight (kg)	Before injection Threshold (cc)	per kg	PTC	After injection Threshold (cc)	per kg	PTC
21	1.8	0.9	0.50	200	2.4	0.75	133
22	1.7	1.3	0.77	131	1.5	0.88	113
23	2.2	1.4	0.64	157	1.6	0.73	137
24	2.8	1.4	0.50	200	1.5	0.54	205
25	1.8	1.0	0.56	180	1.45	0.81	124
Mean	2.0	1.2	0.59	173	1.69	0.74	142

remaining 1 case, the change fell within the range of variation in the same individual.

DISCUSSION

The method for inducing convulsions by metrazol has widely been used since Langelüddeke.[16] Thus, Nakagawa[17] and Matsumoto[18] measured the threshold amount of metrazol for convulsions; Kishi[19] states that the convulsive threshold for the same rabbit did not change markedly within one week or so. This agrees with my findings mentioned

already. The PTC was found convenient to denote the predisposition to convulsions quantitatively.

Several reports are on hand concerning the relation of the functions of the autonomic nerves with the predisposition to convulsions. According to Yakovlev,[3] there occurs a mosaic separation between the sympathetic and the parasympathetic in the premonitory period of an epileptic fit, then the sympathetic overaction predominates, and the fit takes place. Trinker,[4] Gellhorn,[5] and Horiguchi & Takeya[6] report that administration of a large amount of adrenalin facilitates the onset of convulsions by metrazol, insulin and electric shock. It is clear also from my results that adrenalin administration (i.e. causing excessive tone of the sympathetic) increased the predisposition to convulsions. From the results of administering atropine to patients of genune epilepsy, Kanazawa[7,8] concluded that in cases of grand mal and psychomotor seizure this drug is effective for preventing the onset of convulsions, but it may sometimes be rather harmful to other types of cases. Max de Grinis & Scoda[20] state that the drug is efficacious against convulsions, but Hizig[21] reports to the contrary. In the present work, changes in parasympathetic function (due to atropine or pilocarpine) do not seem to show a definite tendency in affecting the predisposition to convulsions. Methonium compounds, sympathetic inhibitors known to us since 1948 (Varlow & Inn[22] and Patson & Zaimis),[23] are said to act also as parasympathetic inhibitors. Chlorpromazine is a double inhibitor of the sympathetic and the parasympathetic, which was first published by Courvoisier[24] as "largactil" and is now widely used in various fields of medicine. But there has histherto appeared no report on the effect of a methonium compound on convulsions. As for chlorpromazine, papers dealing with this drug have been published by David & Benda,[25] Ueki[26] and Kurosawa,[27] but the information is still incomplete. In my experiments of administering these two drugs, they decreased the predisposition to convulsions pronouncedly. In summary, it may be stated that the double inhibition of the autonomic nerves serves to decrease the predisposition to convulsions, which is largely due to the inhibition of the sympathetic.

When the action potentials of the autonomic nerves in normal rabbits were recorded by means of an electroencephalograph, the peak of the frequency histogram lay between 39.2 and 24 cycles per second. This agrees with what has been reported by Orihara,[12] who found that the effects of the former was characterized by a decrease of sympathetic fast waves (39.2 to 24 cycles per second), and those of the latter by a increase of vagal intermediate waves (15.5 to 9.8 cycles per second). Nearly the same results were obtained also in the present investigations. It is interesting that each drug acted on only one type of the nerves selectively.

Furthermore, atropine acted chiefly on the vagus, bringing about a transitory increase in amplitude of the action potential (followed by a later decrease below the normal level), an increase of fast waves (39.2 cycles or more per second), and sometimes a decrease of intermediate waves (15.5 to 9.8 cycles per second). Orihara[12] observed abolition of pilocarpine effects by administration with atropine; he did not test atropine singly, however. Concerning the effects of hexamethonium and chlorpromazine, it is to be noted that the amplitude was decreased markedly in both nerves (especially in the sympathetic); indeed, in 2 cases of hexamethonium administration, the action potential showed a base-line fluctuation. As for the frequency, fast waves (39.2 cycles or more per second) was increased in many cases, the frequency changes in the sympathetic due to hexamethonium were a little different, in that fast waves disappeared with decrease in amplitude and by an increase of intermediate waves (24 to 9.8 cycles per second). Thus, from the changes in action potential, it may be understood that the two drugs acted on both the vagus and the sympathetic. According to Endo[28] and Motokawa,[15] the EEG of normal rabbits mainly consists of slow waves of 2 to 3 cycles per second and fast waves of about 30 cycles per second in most cases. In the present work, however, fast and intermediate waves (39.2 to 15.5 cycles per second) were predominant. Concerning effects of adrenalin, Bartley & Hembecker[29] observed a decrease in amplitude in some cases, but Gibbs & Lennox[30] found no remarkable changes, and Orihara[12] recognized an increase of slow waves. Adrenalin was found to bring about a slight decrease in amplitude and an increase of slow waves (6.2 to 3.9 cycles per second). Pilocarpine is reported by Orihara[12] to increase the amplitude remarkably, while I noticed no such conspicuous increase but rather a base-line fluctuation and an increase of intermediate waves (15.5 to 9.8 cycles per second). Atropine brought about someincrease in amplitude but no noticeable change in frequency. Kanazawa[7,8] noticed, however, in his studies of patients with epilepsy, a contrariety on the changes in the EEG due to atropine: When the frequency was less than the normal, it was brought to nearly the normal level; when the frequency was normal, it reduced. In the cases of hexamethonium and chloropromazine, the amplitude did not change noticeably, but the period showed a remarkable prolongation in that there occurred pronounced increase of intermediate waves (15.5 to 6.2 cycles per second) in the former drug and of intermediate and slow waves (9.8 to 2.4 cycles per second) in the latter. According to Sano[31] and Suwa,[32] these drugs, which have recently come into use as principal chemical agents for artificial hibernation, prolong the period and diminish the amplitude in the EEG under hypothermia caused by these agents. Shio-

zawa[32] reported that this tendency was remarkable beneath the cortex of the cerebrum, being thus suggestive of their effect on the autonomic center in the diencephalon.

The rates of both pulse and respiration, which served as aids for judging the functions of the autonomic nerves, decreased by administration of pilocarpine, hexamethonium, and chlorpromazine, the changes thus reflecting their action as parasympathetic stimulant or sympathetic inhibitor. In cases of adrenalin and atropine, however, the pulse rate did not always run parallel with the rate of respiration, which might possibly due to the production of some substance anatogonizing these agents in the body (Morishima[34]).

Summary

1. Five "autonomic drugs" (adrenalin, pilocarpine, atropine, hexamethonium, and chlorpromazine) were tested, with 25 adult rabbits, for their effects on the predisposition to convulsions and the relations of these effects with the action potentials of the autonomic nerves and the brain.

2. The predisposition of the same rabbit to convulsions remained almost unchanged within a short period of time. In the frequency histogram for each action potential, the peak was always situated between 39.2 and 24 cycles per second.

3. Of the two types of the autonomic nerves, the sympathetic had the largest effect: its overaction increased predisposition to convulsions, and its inhibition decreased it.

4. The "autonomic drugs" used had selective effects on the action potentials of the autonomic nerves:

a) Adrenalin decreased sympathetic fast waves (39.2 to 24 cycles per second).

b) Pilocarpine increased vagus intermediate waves (15.5 to 9.8 cycles per second).

c) Atropine increased vagus fast waves (39.2 cycles or more per second).

d) Hexamethonium and chlorpromazine decreased amplitude and increased fast waves (39.2 cycles or more per second) of both nerves.

5. In electroencephalograms, there were observed an increase of slow waves (6.2 to 3.9 cycles per second) by adrenalin, an increase of intermediate waves (15.5 to 9.8 cycles per second) by pilocarpine, no remarkable change by atropine, and a prolongation of the period by hexamethonium and chlorpromazine. Direct relations with the predisposition to convulsions could hardly be seen.

References

1) Förster, Dtsch. Ztsch. Nervenkr., 1926, **94**, 6.
2) Kessler & Gellhorn, Proc. Soc. Exp. Biol., 1941, **46**, 64.
3) Yakovlev, Arch. Neurol. Psychiat., 1937, **37**, 523.
4) Trinker & Serota, J. Neurophysiol., 1938, **1**, 573.
5) Gellhorn *et al.*, Arch. Neurol. Psychiat., 1939, **42**, 820.
6) Horiguchi & Takeya, Noshinkei Ryoiki (Jap.), 1953, **6**, 192.
7) Kanazawa, *ibid.*, 1953, **6**, 192.
8) Kanazawa, *ibid.*, 1954, **7**, 181.
9) Erlanger & Gasser, cited in references 10) and 12).
10) Sakamoto, Nihon Rinsho (Jap.), 1950, **8**, 1001.
11) Tanaka, Nihon Gekagakkai Zasshi (Jap.), 1953, **54**, 112.
12) Orihara, Tohoku J. Exp. Med., 1952, **57**, 43.
13) Berger, Arch. Psychiat. Nervenkr., 1929, **87**, 527.
14) Wilson, Am. Heart J., 1931, **6**, 637.
15) Motokawa, Noha (Jap.), Nanjoshoten, Tokyo, 1947.
16) Langelüddeke, Dtsch. Med. Wochr., 1936, **62**, 1588.
17) Nakagawa, Shinrinsho (Jap.), 1947, **2**, 134.
18) Matsumoto, Tohoku Igaku Zasshi (Jap.), 1953, **48**, 228.
19) Kishi, *ibid.*, 1956, **53**, 50.
20) Max de Grimis & Scoda, cited in reference 7).
21) Hizig, cited in reference 7).
22) Barlow & Ing, Brit. J. Pharmacol., 1948, **3**, 298.
23) Patson & Zaimis, *ibid.*, 1949, **4**, 381.
24) Courvoisier, Arch. Internat. Pharmacodyn., 1953, **92**, 305.
25) David & Benda, cited in Wintermin (Jap.), Shionogi Pharm. Ind. Ltd., Osaka, Japan, 1955.
26) Ueki, J. Internat. Leag. Eplepsy, 1956, **5**, in press.
27) Kurosawa, *ibid.*
28) Endo, Folia Psychiat. Neurol. Jap., 1949, **3**, 109.
29) Bartley & Hembecker, Am. J. Physiol., 1940, **130**, 13.
30) Gibbs & Lennox, Arch. Inn. Med., 1937, **60**, 154.
31) Sano, 4th Annual Meeting of Japan EEG. Society. 1955, Apr. Osaka, Japan.
32) Suwa, *ibid.*
33) Shiozawa, *ibid.*
34) Morishima, Yakubutsugaku, (Pharmacology.), Nankodo, Tokyo, Japan, 1938.

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Relationships of the Predisposition to Convulsions with the Action Potentials of the Autonomic Nerves and the Brain

II. Changes in Action Potential of the Autonomic Nerves and the Brain under Conditions for Increasing the Predisposition to Convulsions

By

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(Received for publication, June 4, 1956)

With the progress in studies of epilepsy, various methods for inducing convulsions have been devised. The method that is most widely in clinical use still now is to inject metrazol intravenously. On the other hand, causation of metabolic alkalosis, ligation of the carotid artery, administration of excess water, and application of acetylcholine to the cerebral cortex are known to be capable of increasing the predisposition to convulsions. The present paper deals with changes in action potential of the autonomic nerves and the brain caused by metrazol injection and the above four conditions for increasing the predisposition to convulsions.

Experimental

Methods

The animals used were 21 adult rabbits weighing about 2 kg. The methods for recording the action potential and for measuring the predisposition to convulsions were the same as those described in the first report.[1] The measurement of the predisposition was performed after the action potentials under an experimental condition were recorded. The results of these observations were compared with those that had been obtained with the same rabbits before several days.

Results

Effects of intravenous injection of metrazol on the action potentials (Fig. 1)

Five rabbits were injected through an ear vein with 5 per cent metrazol at a slow constant rate of 0.2 cc. per minute. The action potentials after 1 minute (0.2 cc.) and 2.5 minutes (0.5 cc.) from the start of injection

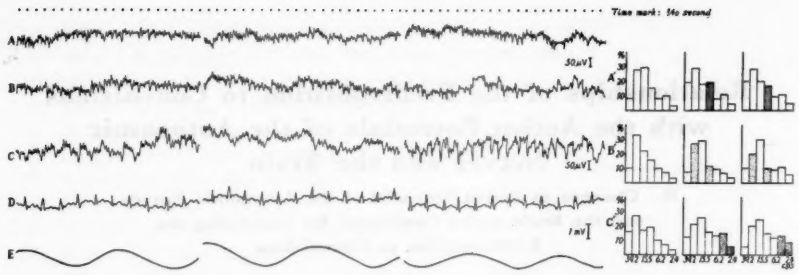

Fig. 1. Effects of metrazol injection on action potentials, etc. From left to right: action potentials before injection, after 0.2 cc. injection, after 0.5 cc. injection, and the corresponding frequency histograms in the same order. *A*, vagus; *B*, sympathetic; *C*, EEG; *D*, ECG; E, respiration; *A'*, *B'*, *C'*: frequency histograms for *A*, *B*, *C*. The explanation of the lettering and other expressions given above is common to all the figure 1, 2, 4, 6, and 8.

were analyzed, with the results as follows: A little after the start of injection (0.2 cc.) there appeared a slight increase of intermediate waves (15.5 to 9.8 cycles per seconds) in the vagus, and a decrease of fast waves (39.2 to 24 cycles per second) and an increase of intermediate waves (15.5 to 9.8 cycles per second) in the sympathetic. These tendencies became more pronounced before the onset of convulsions (0.5 cc.). In the EEG, slow waves (6.2 to 2.4 cycles per second) were increased. The amplitude of each action potential showed a tendency to increase over the normal level. The rates of both pulse and respiration decreased in all the cases.

Effects of alkalosis on the PTC and the action potentials (Figs. 2, 3)

Four rabbits were injected intravenously with 7 per cent sodium bicarbonate solution at a slow, constant rate of 1 cc. per minute. The

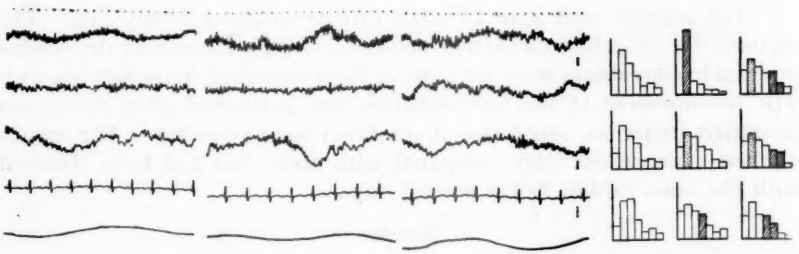

Fig. 2. Effects of alkalosis on action potentials etc. From left to right: action potentials before injection and after injection of 2 cc. and 10 cc. of 7% natrium bicarbonate solution, and the corresponding frequency histograms in the same order. Lettering and other expressions as in Fig. 1.

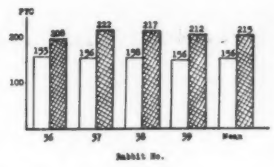

Fig. 3. Effect of alkalosis on PTC. White column: before injection, shaded column: after injection.

action potentials after 2 minutes (2 cc.) and 10 minutes (10 cc.) from the start of injection were analyzed, with the results as follows: The vagus at first showed a transitory increase of fast waves (39.2 to 24 cycles per second) in 3 cases, which were later (10 cc.) decreased below the normal level, and intermediate and slow waves (9.8 to 3.9) cycles per second) were increased instead. The sympathetic showed a decrease of fast waves (39.2 to 24 cycles per second) already in the earlier record (2 cc.), and later (10 cc.), increase of intermediate and slow waves (9.8 to 3.9 cycles per second) in addition. In the EEG, intermediate wave (15.5 to 6.2 cycles per second) increased.

The rates of pulse and respiration at first decreased temporarily; later (10 cc.), the rate of respiration became rather higher than the normal.

The PTC was increased in all cases.

Effects of carotid ligation on the PTC and the action potentials (Figs. 4, 5)

a) Unilateral ligation. Unilateral ligation was performed on 2 rabbits, but it produced noticeable change in neither of the action potentials except for an increase of vagus intermediate waves (15.5 to 9.8 cycles per second) after 5 minutes. The PTC was little affected.

b) Bilateral ligation. Bilateral ligation was performed on 3 rabbits. The action potentials after 1 and 10 minutes of the ligation were analyzed. In the vagus, the increase of intermediate waves (15.5 to 9.8 cycles per second) that was seen in the case of unilateral ligation became more remarkable. In the sympathetic, a decrease of fast waves (39.2 to 24 cycles per second) and an increase of intermediate waves (9.8 to 6.2 cycles per second) was recognized 10 minutes after bilateral ligation. In the EEG, there were shown increases of intermediate waves (15.5 to 9.8 cycles per second) and slow waves (6.2 to 3.9 cycles per second).

One minute after bilateral ligation, the rates of pulse and respiration decreased slightly; after 10 minutes, there were found no marked change except a slight increase in pulse rate.

The PTC remarkably increased in all cases; rabbit No. 44 developed spontaneous convulsions immediately after bilateral ligation.

Effects of intravenous injection of a hypotonic solution on the PTC and the

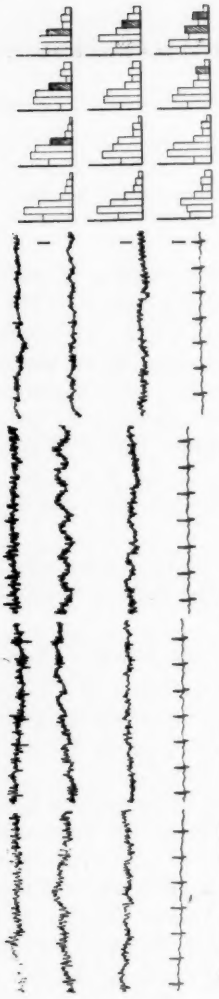

Fig. 4. Effects of carotid ligation on action potentials, etc. From left to right: action potentials before ligation, 5 minutes after unilateral ligation, 1 and 10 minutes after bilateral ligation, and the corresponding frequency histograms in the same order. Lettering and other expressions as in Fig. 1.

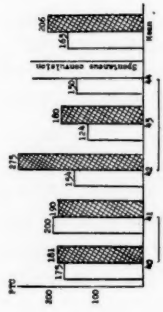

Fig. 5. Effect of carotid ligation on PTC. White column: before ligation, shaded column: after ligation.

action potentials (Figs. 6, 7)

Four rabbits were injected through the femoral vein with 100 cc. of 0.3 per cent saline solution at a slow constant rate of 5 cc. per minute. The action potentials after 12 minutes (60 cc.) and 26 minutes (100 cc.) from the start of injection were analyzed. The effects were more conspicuous in the vagus (an increase of intermediate waves, 15.5 to 9.8 cycles per second); in the sympathetic, no definite changes were seen both

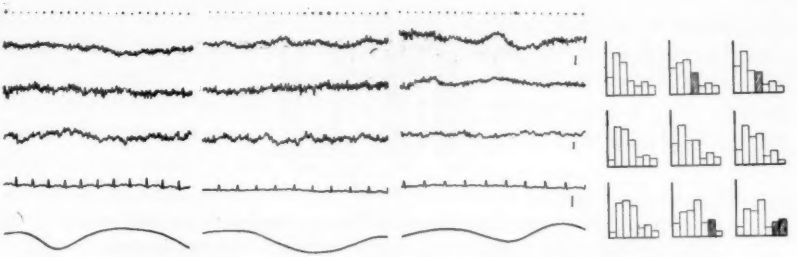

Fig. 6. Effects of administering excess water on action potentials, etc. From left to right: before injection, after injection of 60 cc. and 100 cc. of 0.3% saline solution, and the corresponding frequency histograms in the same order. Lettering and other expressions as in Fig. 1.

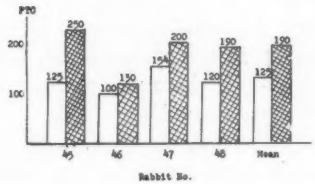

Fig. 7. Effect on PTC of administering excess water. White column: before administration, shaded column: after administration.

in frequency and in amplitude. The EEG changes were characterized by an increase of slow waves (6.2 to 2.4 cycles per second).

The rates of both pulse and respiration were decreased.

The PTC showed an increase in all the cases.

Effects of application of acetylcholine to the cerebral cortex on the PTC and the action potentials (Figs. 8, 9)

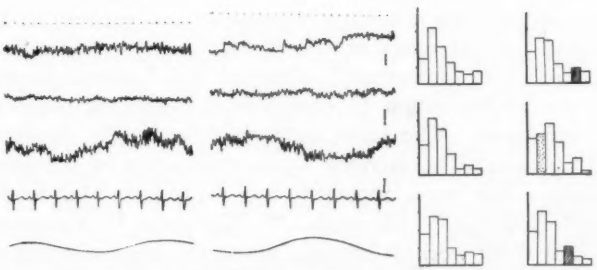

Fig. 8. Effects of acetylcholine application to the cerebral cortex on action potentials, etc. From left to right: before and 5 minutes after application, and the corresponding frequency histograms in the same order.

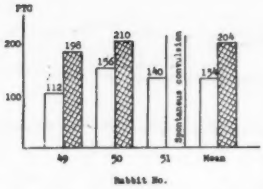

Fig. 9. Effect on PTC of acetylcholine application to cerebral cortex.
White column : before application, shaded column : after application.

A small hole (3 mm. in diameter) was pierced through the skull at the right frontal region, and 0.1 cc. of 10 percent acetylcholine was applied to the cerebral cortex thus exposed. The changes as noticed after 5 minutes were as follows: In the vagus, there were observed a decrease in amplitude, a baseline fluctuation, and an increase of slow waves (6.2 to 3.9 cycles per second); in the sympathetic, fast waves were decreased in all the cases. In the EEG, there was seen an increase of intermediate waves (9.8 to 6.2 cycles per second).

The rates of both pulse and respiration decreased.

The PTC increased conspicuously, and rabbit No. 51 was seized with spontaneous convulsions immediately after application of acetylcholine.

Discussion

Literature which deals with the four conditions that I have chosen here for increasing the predisposition to convulsions is abundant. Thus, according to Rosett[2] and Förster,[3] respiratory alkalosis which was produced in cases of epilepsy induced petit mal. Ninomiya[4] reports that when metabolic alkalosis was caused by administering sodium bicarbonate to patients of genuine epilepsy, it brought about grand mal or ptit mal. By intravenous injection of sodium bicarbonate into rabbits, Kishi[5] lowered the threshold for the onset of convulsions due to metrazol. Sympson & Derbyshire[6] and Matsumoto[7] observed that bilateral carotid ligation increased the predisposition to convulsions, sometimes developing spontaneous convulsions. The latter author[7] refers to the ineffectiveness of unilateral ligation. The increase by overhydration of the predisposition to convulsions has been reported since Wood & Mc. Kibbin,[8,9] by Rowntree,[10] Misawa,[11] Miyagawa,[12] Nonomiya,[4] Kurosawa,[13] and Haga.[14] All these workers brought about the state of overhydration by oral administration of excess water, while Matsumoto[7] injected a hypotonic saline solution intravenously into cats and could increase the predisposition to convulsions. It is reported that acetylcholine has an im-

portant bearing on epileptic convulsions[15]; thus, when it is applied to the cerebral cortex, it increases the predisposition to convulsions by metrazol (Förster,[16] Ito.[17]) According to Sasaki,[18] the predisposition of cats to convulsions by metrazol was increased by injection of acetylcholine into the subcortical nuclei, and its effect was most pronounced when the injection was made in the thalamus.

As for the changes in action potential of the autonomic nerves due to the five conditions for increasing the predisposition to convulsions (including intravenous injection of metrazol alone), it may be summarily said that, except for the case of administering excess water, the changes appeared mainly in the sympathetic, a decrease of fast waves (39.2 to 24 cycles per second) being noticed in the great majority of cases. These resemble the potential changes of the sympathetic due to intravenous injection of adrenalin;[1] this is interesting in view of the fact that adrenalin administration increases the predisposition to convulsions. It should be mentioned, however, that intravenous injection of metrazol, administration of excess water, and carotid ligation brought about also an increase of vagus intermediate waves (15.5 to 9.8 cycles per second) as seen in the case of intravenous injection of pilocarpine. This shows that excessive tone of the sympathetic is not the only agent for increasing the predisposition to convulsions. The rates of pulse and respiration were both decreased by metrazol injection, administration of excess water, and acetylcholine application, while the two rates did not always run parallel with each other in the cases of alkalosis and carotid ligation. From the various facts mentioned above, the conditions for increasing the predisposition to convulsions may be classified into the following types, as for as the five conditions chosen in the present work are concerned: a) sympathetic type (alkalosis and application of acetylcholine), b) sympathetic-parasympathetic type (metrazol injection and carotid ligation), and c) parasympathetic type (administration of excess water). That there appear marked discrepancies in action potential between the sympathetic and the parasympathetic (especially, that the sympathetic is in a state of hyperirritability) may be taken to have an important bearing on the increase of the predisposition to convulsions.

The EEG changes under a condition of increasing the predisposition to convulsions were characterized by an increase of slow waves (6.2 to 2.4 cycles per second) in the cases of metrazol injection, administration of excess water, and carotid ligation, and by an increase of intermediate waves (15.5 to 6.2 cycles per second) in the remaining two cases. Effects on the EEG of intravenous metrazol injection have been studied by many investigators, among whom Takizawa,[19] Matsumoto,[7] Sasaki,[18] and Kishi[5] agree in having observed increase in amplitude, prolongation in

period, and appearance of spikes. In addition, Kishi[5] found that alkalosis in rabbits produced prolongation of the period in the EEG and baseline fluctuation in many cases, while it developed a thalamic EEG of convulsive type in some cases with cats. Matsumoto[17] is of the opinion that bilateral carotid ligation abolished the action potential at the cerebral cortex, and the unbalance thus produced between it and the subcortex may form a cause of the increase in the predisposition to convulsions. Slow waves in the EEG as seen in the case of administration of excess water were ascribed by Matsumoto[7] and Haga[14] to cerebral edema. Sasaki[18] mentions the appearance of spike and dome in the EEG of the cat due to injection of acetylcholine into the thalamus.

Summary

1. It was demonstrated with 21 rabbits that causation of alkalosis, ligation of the carotid artery, administration of excess water, and acetylcholine application to the cerebral cortex were capable of increasing the predisposition to convulsions. Examination was made of the action potentials of the autonomic nerves and the brain under these five conditions (including intravenous injection of metrazol alone).

2. From the changes in action potential of the autonomic nerves, the conditions for increasing the predisposition to convulsions may be classified into three types: sympathetic, sympathetic-parasympathetic, and parasympathetic. Under a condition of increasing the predisposition to convulsions, there is brought about a state of unbalance in action potential between the sympathetic and the parasympathetic, In this respect, the importance of the excessively tonic state of the sympathetic has been emphasized.

3. Causation of alkalosis and application of acetylcholine to the cerebral cortex belong to the sympathetic type, and are characterized by a decrease of fast waves (39.2 to 24 cycles per second) in the sympathetic.

4. Intravenous injection of metrazol and ligation of the carotid artery belong to the sympathetic-parasympathetic type, and are characterized by a decrease of fast waves (39.2 to 24 cycles per second) in the sympathetic and an increase of intermediate waves (15.5 to 9.8 cycles per second) in the vagus.

5. Administration of excess water belongs to the parasympathetic type, and is characterized by an increase of intermediate waves (15.5 to 9.8 cycles per second) in the vagus.

6. The changes in the EEG under conditions for increasing the predisposition to convulsions are characterized by a prolongation of the period and increase of intermediate waves (15.5 to 6.2 cycles per second) and slow waves (6.2 to 2.4 cycles per second).

References

1) Onuma, Tohoku J. Exp. Med., 1956, **63**, in press.
2) Rosett, Brain, 1924, **47**, 293.
3) Förster, Dtsch. Ztsch. Nervenkr., 1925, **83**, 347.
4) Ninomiya, No to Shinkei (Jap.), 1954, **6**, 202.
5) Kishi, Tohoku Igaku Zasshi (Jap.), 1956, **58**, 50.
6) Simpson & Derbyschire, Am. J. Physiol., 1934, **109**, 99.
7) Matsumoto, Tohoku Igaku Zasshi (Jap.), 1953, **48**, 228.
8) Weed & Mc. Kibbin, Am. J. Physiol., 1919, **48**, 512.
9) Weed & Mc. Kibbin, *ibid.*, 1919, **48**, 531.
10) Rowntree, Arch. Int. Med., 1922, **306**, 29.
11) Misawa, Tokyo Igaku Zasshi (Jap.), 1927, **41**, 669.
12) Miyakawa, Kumamoto Igaku Zasshi (Jap.), 1938, **14**, 1767.
13) Kurosawa, Nihon Seishin Shinkei Gakkai Zasshi (Jap.) ,1943, **48**, 231.
14) Haga, Tohoku Igaku Zasshi (Jap.), 1953, **48**, 319.
15) Kanazawa, Noshinkei Ryoiki (Jap.), 1954, **7**, 181.
16) Förster, Am. J. Physiol., 1950, **161**, 430.
17) Ito, Nihon Seiri Gakkai Zasshi (Jap.), 1952, **14**, 60.
18) Sasaki, Tohoku Igaku Zasshi (Jap.), 1955, **51**, 1.
19) Takizawa, *ibid.*, 1953, **48**, 214.

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Responsiveness of the Sweat Glands of a Formosan Monkey to Some Sudorific Substances

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(Received for publication, January 14, 1957)

It has been generally accepted that the secretory nerves to the sweat glands of human beings are sympathetic cholinergic. Nevertheless, it was demonstrated recently by a number of investigators that adrenaline and allied substances produce sweating in man by acting directly on the sweat glands. The minimal effective concentration of adrenaline applied intracutaneously or subcutaneously was 10^{-7} to 10^{-8} (Wada & Takagaki; Kisin; Wada). For DL-noradrenaline, it was 2×10^{-7} (Tanaka & Nakamura). In contrast, the minimal effective concentration for acetylcholine was 10^{-12} (Wada & Takagaki; Kisin) and that for carbaminoylcholine was 10^{-12} (Kisin) and 10^{-12} to 10^{-13} (Tanaka, Koyama & Wada, unpublished).

Evidence has been accumulating which indicates that the sensitivities of the sweat glands to sympathomimetic and parasympathomimetic sudorific agents differ between species.

In view of this, the minimal effective concentrations of the following sudorific agents for eliciting local sweating in a female Formosan monkey (Macacus cyclopis), weighing 1.75 kg., were determined.

Chlorides of L-adrenaline (Sankyo), DL-noradrenaline (Sankyo), acetylcholine (Roche) and carbaminoylcholine (Merck) were dissolved in 0.9% NaCl solution in graded concentrations, which were given in terms of their bases, and 0.1 ml. of each solution was injected intracutaneously to the shoulder of the animal, under non-anaesthesia. For visualizing sweating, the iodine-starch method of Wada & Takagaki was used.

The minimal effective concentrations thus estimated were 10^{-6} L-adrenaline, 2×10^{-4} DL-noradrenaline, 10^{-4} acetylcholine and 10^{-7} carbaminoylcholine.

References. Kisin, Chem. Abstr., 1949, **43**, 2323; Tanaka & Nakamura, Kumamoto Med. J., 1955, **8**, 26; Wada, Science, 1950, **111**, 376; Wada & Takagaki, Tohoku J. Exp. Med., 1948, **49**, 284.

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Relationships of the Predisposition to Convulsions with the Action Potentials of the Autonomic Nerves and the Brain

III. Changes in Action Potential of the Autonomic Nerves and the Brain under Conditions for Decreasing the Predisposition to Convulsions

By

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(Received for publication, June 4, 1956)

The therapy of epilepsy had extensively been studied from pharmaceutic, surgical, and other various angles. In an earlier paper (second report)[1] I published a study concerning the changes in action potential of the autonomic nerves and the brain under conditions for increasing the predisposition to convulsions. The present paper (third report) deals with the potential changes under conditions for decreasing the predisposition to convulsions, as compared with the normal potentials. For obtaining such conditions, the following three relatively easy experimental means were used: anesthetization with baribiturate, causation of ketosis, and destruction of the thalamus on each side.

Experimental

Methods

Ten adult rabbits weighing about 2 kg. and four adult cats were used: the former, for experiments of barbiturate anesthetization and causation of ketosis; the latter, for those of thalamic destruction. Thalamic destruction was carried out in the following manner: A cat was fixed in a prone position and submitted to craniotomy; a Horsley-Clark apparatus[2] was then adapted to the head, and 0.1 cc. of a 1:10,000 dilution of corrosive sublimate was injected, under the guidance of Gerard's chart,[3] into the thalamus (ventral portion of the nucleus lateralis thalami) on each side. The cat was killed after completion of the experiment, and serial sections of the cerebrum were prepared to check the actual position of the injection. The methods for recording the action potentials and of measuring the predisposition to convulsions were the same as those

described in my earlier papers (first and second reports.[1),14)])

Results

Action potentials of the autonomic nerves and the brain, the rates of pulse and respiration, and the predisposition to convulsions in normal cats (Fig. 1; Tables I, II)

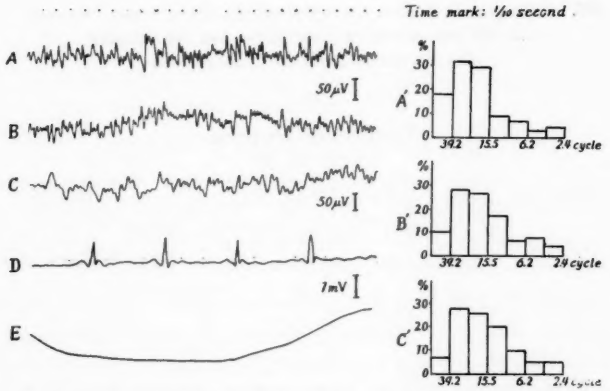

Fig. 1. Spontaneous action potentials and their frequency histograms of a normal cat.

A, vagus action potentials; B, sympathetic action potential; C, EEG; D, ECG; E, respiration; A′, B′, C′: frequency histograms for A, B, C. The explanation of the lettering and other expression give above is common to all the figure 1, 2, 4, and 6.

TABLE I

Rates of Pulse and Respiration of Normal Cats

No.	Pulsation per minutes	Respiration per minutes
1	125	25
2	133	26
3	150	28
4	145	30
Mean	138	27

TABLE II

Predisposition to Convulsions by Metrazol of Normal Cats

No.	Weight (kg)	Convulsive threshold (cc)	per kg	PTC
1	2.2	2.8	1.30	76.0
2	2.1	3.2	1.54	65.0
3	1.8	1.8	1.00	100.0
4	2.0	2.4	1.20	82.0
Mean	2.0	2.5	1.26	80.7

Four adult cats were used. The amplitude of the action potential was 30 to 50 μV both in the vagus and in the sympathetic, and the peak of the frequency histogram lay in a range between 39.2 and 24 cycles per second in both. Thus, it will be seen that the action potentials of the cat were essentially the same as those of the rabbit except for intermediate

waves (24 to 15.5 cycles per second), which occurred at a little higher rate. The rate of pulse was 125 to 150 (average: 138.3) per minute, and that of respiration 25 to 30 (average: 27.3), both rates being much lower than those of the rabbit. The PTC showed a value much lower (65 to 100, average: 80.7) than in the rabbit.

Effects of barbiturate anesthetization on the PTC and the action potentials (Fig. 2, 3)

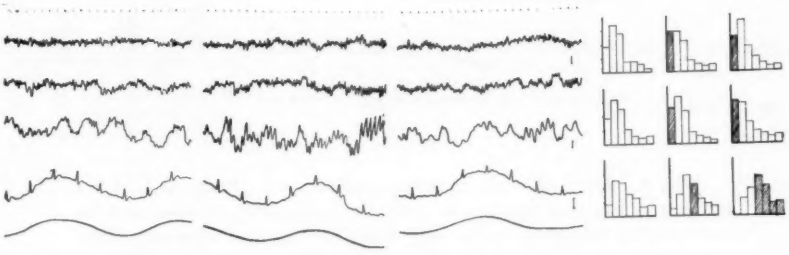

Fig. 2. Effects of barbiturate anesthesia on action potentials, etc. From left to right: before injection, after injection of 5 cc and 10 cc. of 0.5% thiopental sodium, and the corresponding frequency histograms in the same order, lettering and other expressions as in Fig. 1.

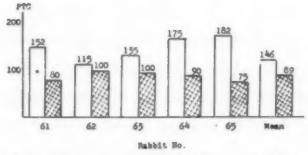

Fig. 3. Effect of barbiturate anesthesia on PTC. White column: before injection, shaded column: after injection.

Five rabbits were slowly injected through an ear vein with 25 mg. of 0.5 per cent thiopental sodium per kg. body weight at a constant rate of 1 cc. per minute. The action potentials after 5 minutes (5 cc.) and 10 minutes (10 cc.) from the start of injection were analyzed, with the following results: Both the sympathetic and the vagus showed an increase of fast waves (39.2 cycles or more per second) already after 5 minutes (5 cc.); after 10 minutes (10 cc.), the amplitude was decreased in all cases. In the EEG, there was noticed an increase in amplitude as well as an increase of intermediate waves (15.5 to 9.8 cycles per second) already after 5 minutes (5 cc.); after 10 minutes (10 cc.), no fast waves (39.2 cycles or more per second) were seen, and intermediate waves (15.5 to 6.2 cycles per second) and slow waves (6.2 to 2.4 cycles per second) increased instead.

The rate of pulse showed a gradual decrease, while the rate of re-

spiration presented a slight increase (after 5 minutes) followed by a gradual decrease in process of anesthetization.

The PTC showed a decrease in all cases.

Effects of ketosis on the PTC and the action potentials (Figs. 4, 5)

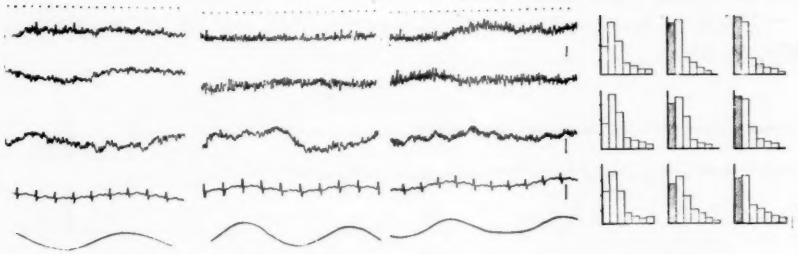

Fig. 4. Effects of ketosis on action potentials, etc. From left to right: before injection, after injection of 1 cc. and 2 cc. of 50% acetone, and the corresponding histograms in the same order, lettering and other expressions as in Fig. 1.

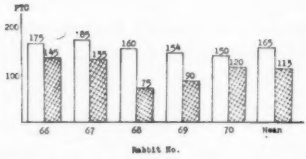

Fig. 5. Effect of ketosis on PTC. White column: before injection, shaded column: after injection.

Five rabbits were slowly injected through an ear vein with 50 per cent acetone at a constant rate of 1 cc. per 5 minutes. The action potentials after 5 minutes (1 cc.) and 10 minutes (2 cc.) from the start of injection were analyzed: Already after 5 minutes, there was seen in all cases an abnormal increase of fast waves (39.2 cycles or more per second) both in the autonomic nerves and the brain; after 10 minutes, this tendency was more remarkable, while a decrease of intermediate waves (15.5 to 9.8 cycles per second) was noticed in the sympathetic in 4 cases.

The rates of pulse and respiration had a tendency to incarease in proportion to the amount injected.

The PTC decreased in all cases.

Effects of bilateral thalamic destruction on the PTC and the action potentials (Figs. 6, 7)

Four cats were submitted to destruction of the thalamus on each side. The action potentials after 5 and 10 minutes after bilateral destruction were analyzed. After 5 minutes, the amplitude became so

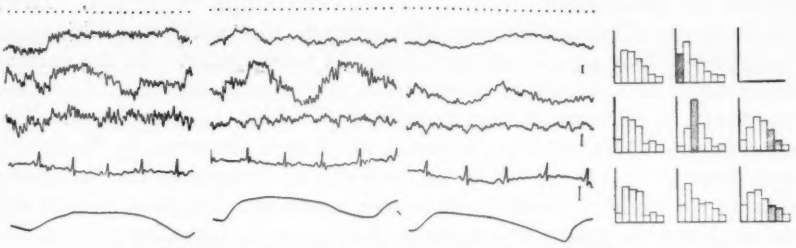

Fig. 6. Effects of thalamic destruction on action potentials, etc. From left, to right: before, 5 and 10 minutes after bilateral thalamic destruction, and the corresponding histogram in the same order, lettering and other expressions as in Fig. 1.

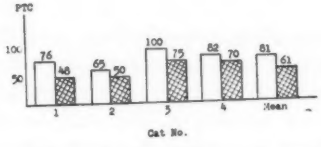

Fig. 7. Effect of thalamic destruction on PTC. White column: before destruction, shaded column: after destruction.

low that the period was immeasurable. In the sympathetic, there were seen, after 5 minutes, a base-line fluctuation in all cases and an increase of intermediate waves (24 to 15.5 cycles per second); after 10 minutes, the amplitude was markedly decreased and intermediate and slow waves (9.8 to 3.9 cycles per second) were increased. In the EEG, there were observed a gradual prolongation of the period in process of time and increases of intermediate and slow waves (9.8 to 3.9 cycles per second).

The pulse rate decreased, but the rate of respiration showed no remarkable changes.

The PTC decreased in all cases.

Discussion

With the progress of studies in epilepsy a large number of anticonvulsants have been published. And especially, the efficasy of barbiturate was clearly demonstrated in experiments by Matsumoto[4]; its antagonistic action on metrazol have been pointed out by Johnes,[5] and Takizawa and Matsumoto.[6] My experimental results also clearly show the efficacy of this agent for decreasing the predisposition to convulsions. Moreover, Sasaki[7] and Kahata[8] found electroencephalographically that among various subcortical nuclei the thalamus was affected by barbiturate most inhibitorily. According to Kishi[9] an increase of ketones in the blood de-

creases the predisposition to convulsions, independent of the pH. Thus, the treatment of cases of epilepsy with ketogenic diet, which was introduced by Wilder[10] long ago, is still now in use in some circles. In the present experiments also, ketosis as produced by intravenous injection of acetone was found to decrease the predisposition of rabbits to convulsions. The conception of "highest level seizure," which was advanced by Penfield[11] and Jasper,[12] is considered to be the focus of epilepsy in the thalamus; thus, in our country, Wada[13] and Sasaki[7] made experiments in view of this point: From the results of systematic destruction by corrosive sublimate of various nuclei in the extrapyramidal system, the latter author[7] reached the conclusion that bilateral thalamic destruction is most effective for decreasing the predisposition of convulsions. The results obtained in the present work were essentially the same.

Concerning the action potentials of the autonomic nerves, the results obtained may be summarized as follows: In the conditions of barbiturate anesthesia and ketosis there was seen an increase of fast waves (39.2 cycles or more per second) both in the vagus and in the sympathetic; especially in the former condition, the amplitudes of both became decreased. These findings resemble those obtained in the case of chlorpromazine administration.[14] From the fact that these conditions do decrease the predisposition to convulsions, it may easily be concluded that the action potentials of both the sympathetic and the vagus reflect inhibitory effects on both nerves. Cats were used instead of rabbits for experiments of bilateral thalamic destruction. As the autonomic nerve action potentials of the cat were not found to be fundamentally different from those of rabbits, and are characterized by a remarkable decrease in amplitude as in the case of rabbits, the changes as shown in these cases also reflect inhibitory effects on both nerves. As for the frequency, there were seen increases of vagus fast waves (39.2 cycles or more per second) and of sympathetic intermediate waves (24 to 15.5 cycles per second); these changes also were similar to those caused by administration with hexamethonium.[14] Thus, it may be said summarily that the above-mentioned three conditions for decreasing the predisposition to convulsions have inhibitory effects both on vagus and on the sympathetic.

In the EEG, Matsumoto[4] and Kahata[8] have recently demonstrated the appearance of so-called "barbiturate burst" (i.e. increase in amplitude directly after intravenous injection of barbiturate, as was already observed by Blecher, Donough & Fobes.[15]) Kahata[8] reports a decrease in amplitude and a prolongation in period with progress of anesthesia. In my experiments also there was seen a tendency toward gradual increase of slow waves with progress of anesthesia. In my experiments also there was seen a tendency toward gradual increase of slow waves (15.5 to 9.8

cycles per second) were increased in early stages of anesthesia. Concerning effects of ketosis on the brain potential of rabbits, Kishi[9] points out a decrease in amplitude and a shortening in period; in my experiments, however, no marked change was seen in amplitude, though the period was conspicuously shortened (fast waves of 39.2 cycles or more per second being increased). These changes are different from those due to anesthesia (Abe,[16] Kahata,[8] and others) and from those due to acidosis (Ninomiya[17]), and may be ascribed to the effect of ketosis alone. The effects of thalamic destruction is reported by Sasaki,[7] who injected metrazol soon after thalamic destruction, and observed changes appearing in the EEG. In my experiments, there were seen, after bilateral thalamic destruction, a decrease in amplitude, a prolongation in period, and increases of intermediate waves (9.8 to 6.2 cycles per second) and slow waves (6.2 to 3.9 cycles per second).

The rates of pulse and respiration were decreased by barbiturate anesthetization and thalamic destruction, but increased by ketosis, thus showing no definite tendency.

Summary

1. With 10 rabbits and 4 cats it was demonstrated that the predisposition to convulsions were decreased by the following three conditions: barbiturate anesthetization, causation of ketosis, and bilateral thalamic destruction. Changes in action potential of the autonomic nerves and the brain under these conditions were examined.

2. There were observed no fundamental differences between the cat and the rabbit in action potential of the autonomic nerves and the brain, and the frequency histogram for each action potential had its peak in the range between 39.2 and 24 cycles per second in all cases.

3. Barbiturate anesthesia and ketosis brought about an increase of fast waves (39.2 cycles or more per second) in the action potentials of the vagus and the sympathetic, while thalamic destruction and barbiturate anesthesia caused a decrease in amplitude. It has been concluded that these predisposition-decreasing conditions act on both the vagus and the sympathetic in an inhibitory manner.

4. As for the brain potential, there was seen no definite tendency: Barbiturate anesthesia and thalamic destruction prolonged the period, while ketosis shortened it.

References

1) Onuma, Tohoku J. Exp. Med., 1956, **63**, in press.
2) Horsley & Clark, Brain, 1908, **31**, 45.
3) Gerard *et al.*, Arch. Neurol. Psychiat., 1936, **36**, 711.

4) Matsumoto, Tohoku Igaku Zasshi, (Jap.) 1953, **48**, 228.
5) Johes & Murphey, J. A. M. A., 1950, **143**, 884.
6) Takizawa & Matsumoto, Masui (The Japanese Journal of Anesthesiology), 1952, **1**, 171.
7) Sasaki, Tohoku Igaku Zasshi, (Jap.), 1955, 51, **51**, 1.
8) Kahata, Masui (The Japanese Journal of Anesthesiology), 1953, **2**, 91.
9) Kishi, Tohoku Igaku Zasshi, (Jap.), 1956, **53**, 50.
10) Wilder, Mayo Clinic Bull., 1921, **2**, 307.
11) Penfield, EEG Clin. Neurophysiol., 1949, **1**, 349.
12) Jasper *et al.*, Epilepsy, 1937, Sect. IV, 27. W. & Wilkins.
13) Wada, Folia Psychiat Neurol. Jap., 1951, **5**, 61.
14) Onuma, Tohoku J. Exp. Med., 1956, **63**, in press.
15) Blecher *et al.*, J. Neurophysiol., 1938, **1**, 324.
16) Abe, Masui (The Japanese Journal of Anestheology), 1956, 5 in press.
17) Ninomiya, No to Shinkei, (Jap.), 1954, **6**, 202.

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A New Method of Vital Staining of Fat

By

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The method of vital staining of fat is looked upon as a greatly effective one in studying the nature of fat cells, and especially in studying the relation between these and RES cells.

As to this method, there have been two methods; one is to give powder of Sudan III or Scarlet Red together with feed per os (Jöel & Schönheimer[1]), and the other is to inject solution in alcohol intravenously (Jacobsthal[2]). But these methods are too independable to be applied to the above mentioned purpose. The authers, after various investigations on the method of vital staining, succeeded in creation a satisfactory method, and we will publish its summary as follows.

Experimental

The animals used in the experiments are 200 mice weighting about 20 g.

Test of fat staining dyes. Water solble dyes: Nile Blue has a very strong toxity. 2 mice injected intravenously with 0.2 cc. of 1% solution of this dye in physiological saline died instantly. Rhodamin B is discharged quite rapidly after the injection. When 0.5 cc. of saturated solution of this dye (ca. 0.25%) in physiological saline was injected intraperitoneally, the whole cases died within twelve hours after the injection. From these results, it is clarified that both of the two staining dyes can not serve as the dye we wished to be. Fat soluble dyes: In order to use these dyes, at first the solution in water of these must be obtained. Up to this time there are alcohol, saponin, benzoic caffeine, trichlor acetic acid and soap as solvent of these dyes, but all being strong in toxity, and weak in their solubility, they can not be used. The authors, thinking of the fact that surface active agents of Tweens and Spans have the natures of both hydrophilic and lipophilic in themselves (Mima[3]), made experiments by using, in single or mixed way, the highest hydrophilic Span 95, the highest lipophilic Tween 20 and Tween 80 which ranks between

the two formers. By this result, we learned that from the view point fo toxity, actions of local stimulation, and solubility, the single use of Tween 80 is simple and most suitable. Sudan III, Scarlet Red and Oil Red 0 have 0.5% solubility for Tween 80, but only Sudan Black B (SBB) has 5% solubility.

The solutions of these dyes in Tween 80 can be diluted to any density at will by adding physiological saline to it.

Method of injection. 1) Density of solution of staining dyes. When 0.05 to 0.1% solutions of Sudan III or Scarlet Red in physiological saline were injected intraperitoneally, in the whole case (21 cases), the precipitation of the dyes and the adhesion of serous membranes could be seen, but the grade of coloration was too slight. In order to avoid such side-reactions, these dyes should be diluted below 0.025%. Studying on 0.1, 0.25, 0.5, 1.0, 2.5 and 5.0% solutions of SBB, we understood that 1.0 to 5.0% of the solutions bring about more or less the adhesion of peritoneal serous membranes and also that 0.5% of the solution does this sometimes and 0.1% solution brings too slight coloration.—that is, it is discovered that the best density of SBB is 0.25%.

2) Quantity and times of injection. 0.2, 0.5 and 1.0 cc of 0.25% SBB solutions in physiological saline was injected intraperitoneally once or twice every day or every other day—in total from once to fourteen times and the degree of dying was examined in each case. And at the same time the degree of stimulation was tested by seeing whether or not there existed adhesion of the serous membranes and leucocytic reaction of peripheral blood.

The injection of 1.0 cc. SBB solution, regardless of its density, give the mice too much load and brings about excessive accumulation of ascites and slight exhaustion; and 0.2 cc. proves too little. And in the case of the twice-a day-group (9 cases), the adhesion of serous membranes was witnessed in the victims injected the second time.

Accordingly we know that about 0.5 cc. is fit for one time amount of injection, and the time of injection must be limitted to once a day. But the amount of injection should be properly arranged according the weight of a mouse. If 0.5 cc. is injected once a day into a mouse of less than 15 g. in weight adhesion of serous membranes is witnessed sometimes, but when a mouse is more than 25 g., though the injection continues for five days in succession, dying is unsatisfactory. In case a mouse of 20 g. used, a satisfactory result is gained. Therefore we decided that the amount of one injection is to be 0.025 cc. per gram.

In the instances of injecting, once a day, 0.25% SBB solution, in mice at the rate of 0.025 cc. per gram, 5～7 days successive injection shows the best result. When we, after the injection of the solution, take out the

ascites consecutively and examine it referring to the supravital preparation, the ascites looks slight sticky blue till about 24～36 hours, and besides various sizes of floating separate drops of deep blue, various SBB dyed balls can be seen inside of the phagocytic cells. But after 48 hours the dye of the ascites gets remarkably dims or fades away, and its nature and shape return to the original serous again. And the SBB stainable granules in phagocytic cells also dwindle and are discolored. Accordingly perparation should be performed 48 hours after the injection.

In the case of Sudan III, 0.5 cc. of 0.025% solution (1 volume of Sudan III, 200 volumes of Tween 80 and 3800 volumes of physiological saline) is injected once a day for 10 consecutive days and on 12th day a satisfactory result is obtained at any rate.

3) Amount of Tween 80. We prepared two different solutions of 0.2% of SBB; one made of 1 volume of SBB, 20 volumes of Tween 80 and 3800 volumes of physiological saline and the other 1 volume of SBB, 40 volumes of Tween 80 and 360 volumes of physiological saline. When we injected each of these two kinds intraperitoneally respectively for 5～7 days, and compared the results, we found no difference in the degree of coloration. But in the cases injected the former accumulation of dense ascites and sometimes adhesion of serous membranes were seen. So it is desirable that the amount of Tween 80 is small as possible as.

4) Part of injection. Injection can be done intraperitoneally, intravenouslly and subcutaneously. The intraperitoneal injection stains fat tissues in the greatest extent. The injection in vein is far inferior in the point of degree of staining, but serves conveniently for the purpose of studying of attitudes of the fat cells in the organs and RES cells for this dying. And when 0.5 cc. of 0.25% SBB solution is directly injected once into the subcutaneous connective tissue, the fat cells of that part assume deep enough color, without any side-reaction discernible.

Use together with the methodes of vital staining for RES cells. We could at the same time, observe the attitude of RES cells, by injecting intraperitoneally once a day lasting 5 days, 0.5 cc. of the solution prepared by mixing Lithioncarmine at the rate of 0.5～1.0% with 0.25% SBB solution, and by killing the victims on seventh day. When 1.0% Lithioncarmine at the rate of 0.5～1.0% with 0.25% SBB solution and by killing the victims on seventh day. When 1.0% Lithioncarmine was added, the grade of coloration of RES cells in the parenchymatous organs was higher than when 0.5% Lithioncarmine was added; but the animals got tired and the adhesion of intraperitoneal serous membranes was easily done.

Preparation. To make fresh preparations, in case of blood and fluid of serous cavities, a drop of each is put on the object-glass; and in case

of omentum, mesentrium, pleura and subcutaneous tissue, each of them is spread quickly on the object glass; and in case of parenchymatous organs, physiological saline is dropped on it just after it was imprinted. When these processes have been completed, these object-glasses are covered with cover-glasses and seated around with vaseline. And then we look into them. At this time you may use the method of supravital staining with Neutral Red and Janus Green.

To make permanent preparation, in case of blood and fluid of serous cavities, after smear preparation have been made, they are fixed instantly with formalin-steam or with formalin-alcohol (1:4). The same in case of making organ-imprinted preparation. In case of omentum and mesenterium such as require spreading, they must be first fixed in 10% of neutral-formalin, and then are spread again on the object-glasses.

To make counter-staining, in case of simple staining of SBB, 1~2% Alauncarmine staining, and in case of the simple staining of Sudan III and the mixed staining of Carmine SBB, Hematoxylin staining are suitable.

To make microtomized preparations after fixing by 10% of neutral-formalin, and imbedding in Carbowax and microtomizing, counter-staining is made by the method above mentioned.

Rubber syrup is used as seal. At this time you must take care, because intracellular fat drops will run out under the pressure given upon it.

Result

When we injected intraperitoneally 0.25 cc. per gram in weight of 0.25% solution of SBB in physiological saline, once a day for 5 days in succession, and killed the victims on the 7th day, we found that the fat tissues of whole body were dyed out selectively and comparatively evenly with degree convenient for the observation, without causing discernibly stimulating reaction. At this time, the fat tissues of the injected location and the mediastinum were dyed out in the strongest color, while the grade of coloration of other fat tissues, especially the subcutaneous fat tissue is a bit inferior. If you inject the same for over 13 days and killed the victims on the 15th day, the fat tissues of the injected location shows over staining, but the grade of coloration of the subcutaneous fat tissue increases far greater.

The macrophages around the fat tissues are always obviously phagocyting the granules of dyes. These granules are very small and deeply dyed and are very different, in shape and nature, from those of the fat tissues. In the cases of adding Carmine, these macrophages are dyes completely positively. We want to write another article minutely on the subject of the results of each staining of fat cells, and the relation between RES cells and fat cells.

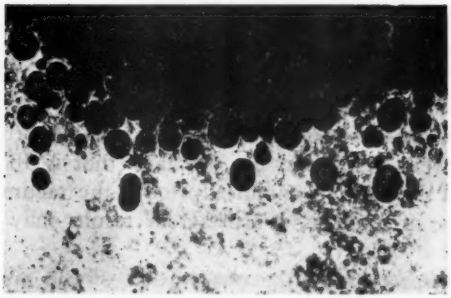

Fig. 1. Strech preparation of omentum of mouse vitaly stained with the Sudan Black B solution. Fat in fat cells has been stained as gross black droplet, whereas that of histiocytes stained fine granular. (about 120×).

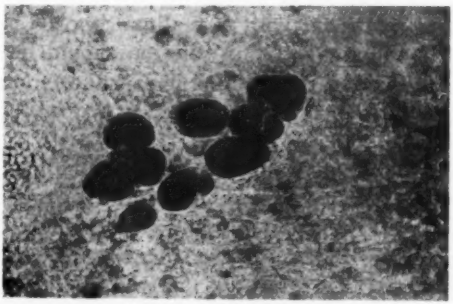

Fig. 2. Strech preparation of mesenterium of mouse vitaly stained with the Sudan Black B solution. The fat droplets in cytoplasm of fat cells have been stained black selectively. (about 140×).

On other subjects: This staining in question proves to be a powerful and effective method not only in selecting the fat cells but also in conducting the cytological study of various kinds of cells.

Technique

Based on the results of various experiments above, the following technique is recommonded as the most rational and practical method.

Preparation of dying solution. i) Place 0.1 g. of Sudan Black B in a large-size test tube, accumulate, 2 cc. of Tween 80 in it, stir it throughout at room temperature, and boil it for about 3 minutes in the double hot water bath while stirring it still. Then Sudan Black B melts completely in Tween 80 and 5% solution of Sudan Black B in Tween 80 can be obtained. ii) To this mixture, and 38 cc. of sterilized physiological saline little by little while stirring it, and boil it on for another 5 minutes. iii)

Filtration after cooling and store it at room temperature. For use, warm it to the bodily temperature of the subject.

Method of dying. Mice were daily injected the above-mentioned solution intraperitoneally with 1 or 2 cc. syringe at the rate of 0.025 cc. for 1 g. weight, for consecutive 5 days; and on the 6th day were left untreated; and were bictimized on 7th day (that is 48 hours after the 5th injection). If the ascites punctured prior to the victimization is serous and have no trace of staining, a satisfactory results may be expected on the whole.

Method of observation. In the observation of the organs and tissues vitally stained on the basis of the afore said method of dying, it is most desirable that fresh or supravital-staining preparations are depended upon, but also the observation according to permanent preparation is possible, too. In this case, smear preparations should be fixed with formol vapor or instantaneously fixed with formalin-alcohol (1 :4).

Otherwise, 10% neutral-formalin solution should be used for fixing, then the stained specimens are imbedded in Carbowax and microtomized. To make strech preparations, first fix in 10% neutral-formalin and then apply the streching.

As the counter-staining, Alauncarmine or Hemalaun staining is used formally according to the color used, rubber syrup are used for sealing.

Results. The intracellular fat droplets of the whole body are stained deep-blue selectively, without causing mentionable side-reactions. By adding Lithioncarmine 0.2 g. (0.5% of the whole solution) to the above-mentioned dying- solution, the relation between the fat cells and RES cells can be more minutely observed.

Summary

We worked out this method of vital staining for fat and showed its method in a concrete way. We think this method of staining to be very much serviciable for the research of the nature of fat cells, especially their relation to the RES cells.

References

1) Jacobsthal, Verh. dtsch. path. Ges., 1909, **13**, 380.
2) Jöel & Schönheimer, Zblt. allg. Path. u. path. Anat., 1924, **34**, 625.
3) Mima, Kagaku-no-Ryoiki (Jap.), 1952, **7**, 23.

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Effects of Mechanical Pressure upon Electrical Excitability of Human Eye

By

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Introduction

In ophthalmotonometry certain mechanical pressure is usually applied to the eyeball. It is well known that under mechanical pressure visual function such as visual acuity, photosensitivity, etc. is lowered or abolished because of impaired circulation of the retina. The effect is, however, only temporary when pressure is moderate and of short duration. This temporary effect may be utilized for investigation of the retinal mechanism of vision, because it is certain that the central mechanism is not involved in the effect.

In the present paper the effect of mechanical pressure applied to the eyeball was investigated upon the electrical excitability of the human eye.

Measurements of electrical excitability were done by Bourguignon & Dejean[1] in their investigation on the chronaxy of the human eye. Motokawa[2-4] used the method of electrical stimulation in combination with photic stimulation for analyzing retinal processes, and a similar method of electric flicker was applied by Motokawa & Suzuki[5] for measuring fatigue. In all these measurements it seems very difficult to rule out the possibility of involvement of the central nervous system, because the index for the measurements was a subjective sensation of electrical phosphene.

As will be shown below, the present investigation has fully confirmed the idea of the previous authors that the seat of electrical stimulation is the retina itself.

Experimental

Method

1. Pressure apparatus

A special pressure apparatus devised for the purpose was applied

to one eye to be examined and the other eye was shielded during the experiment. The apparatus consisted of an eye-disc, A, a spring, B, and a cylindrical holder, F, of the spring. The eye-disc was attached to one end of the spring, while the other end might be clamped into any position of the cylinder by means of a clamp, E. The holder was held in place

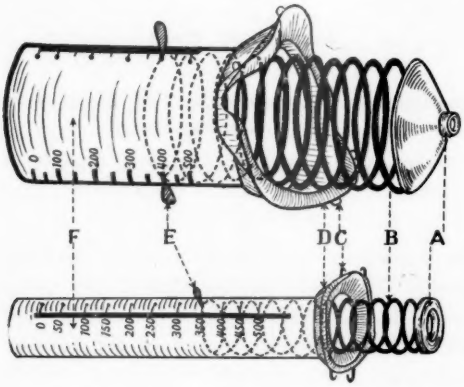

Fig. 1. Pressure apparatus. A: eye-disc, B: spring, C: hook for fixation band, D: impressed model of acrylic resin, E: clamp or stop of spring, F: cylindrical holder of spring. Upper apparatus for greater visual field. Lower one for usual use.

in such a way that the subject could look at a target through the cylindrical pipe of the holder, where the eye-disc was in tight contact with the half-opened lids. The one end of the cylindrical holder was fitted to the face of the subject so well that no dislocation took place between the eye-disc and the lids, and between the holder and the face, either. The satisfactory fitness of the holder to the face was secured by means of an impressed model, D, of acrylic resin. The pressure could be controlled by shifting the clamp, E, along the longitudinal axis of the cylinder. Pressure for each position of the clamp was calibrated by a balance.

In most experiments an apparatus of smaller size, 25 cm. in length and 3 cm. in diameter was used, but the visual field for this apparatus was so small that it was not suitable for investigation of retinal peripheries. In such cases another one of larger size, 20 cm. in length and 10 cm. in diameter was used.

2. Measurements of electrical excitability of the eye

The arrangement for stimulation of the eye was the same as that used by Motokawa,[6] but the method for threshold determination was different; Motokawa and others used to determine the threshold, descending

from a high voltage to the threshold one, but in the present investigation the stimulating voltage was graded in the reverse direction, that is, from a subthreshold one to the threshold. I found that this method was as easy as the previous one, once the subject became accustomed to the procedure, and that the determination was not complicated with multiple thresholds which could be encountered in the previous method, as was reported by Motokawa[6] and Motokawa & Isobe.[7] By the ascending method only one threshold, the lowest one, is measured. The stimulating voltage V was controlled by a resistance, R, and could be expressed in terms of R as follows:

$$V=\frac{12\times 200}{200+R} \text{ volts}$$

Some protocols are shown in Table I.

The stimulating voltage was raised step by step till the subject reported some vague sensation and demanded comparison procedures, in which the stimulus in question was compared with the one of zero-voltage.

Failure in discrimination of the two stimuli is denoted by (−), and success by (+). Simple plus signs mean that the subject could perceive an electrical phosphene without comparison procedure.

TABLE I

Example of Protocols for Determination of Electrical Thresholds of the Eye

H. H.			T. K.			M. W.		
Resistance R in ohms	Answer	Words of subject	Resistance R in ohms	Answer	Words of subject	Resistance R in ohms	Answer	Words of subject
6000	−	No phosphene	8000	−	No phosphene	4000	−	No phosphene
5000	−		7000	−		3000	−	
4000	−		6000	−		2000	−	
3500	−		5900	−		1900	−	
3400	−		5800	−		1880	−	
3300	−		5700	−		1860	−	
3200	−		5600	−		1840	−	
3100	−		5500	±	Not clear	1820	±	Not clear
3000	±	Not clear	5450	(−)		1800	(−)	
2980	(−)		5400	(−)		1780	(−)	
2960	(−)		5350	(−)		1760	(−)	
2940	(+)	Clear	**5300**	(+)	Clear	1740	(−)	
2920	+		5250	+		**1720**	(+)	Clear
2900	+		5200	+		1700	+	
						1680	+	

Results

1. Dependence of electrical excitability of the eye on the mechanical pressure applied to the eyeball

Changes in electrical excitability of the eye were measured during and after application of pressure to the eyeball.

The results are illustrated in Fig. 2, in which reciprocals of thresholds in percentage of the resting value are plotted against pressure in grams. In the inset a frontal aspect of the eye-disc used is shown. The temporal

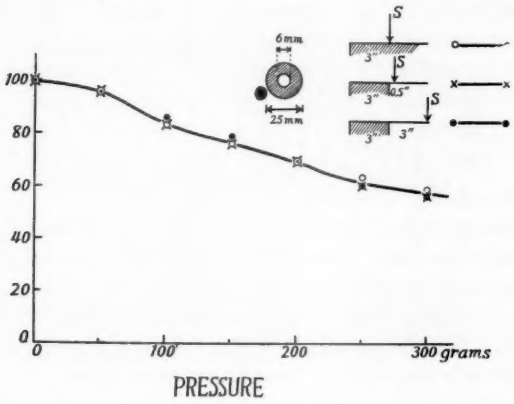

Fig. 2. Relation between pressure and electrical excitability expressed in percentage of resting level. Frontal aspect of eye-disc is shown in inset. Temporal sequence of procedure is also shown in inset. Pressure: hatched. Electrical test stimulus is indicated by an arrow.

relation between pressure and the test stimulus, S, is also shown. In one series of experiments the test shock was applied 3 seconds after the onset of pressure, which was removed after the subject's response. In the second series the test shock was applied 0.5 seconds after 3 seconds' application of pressure, and in the third the interval between the termination of pressure and the test stimulus was 3 seconds. As can be seen in the figure, no difference could be found among the results obtained in these three series.

In all cases the electrical excitability decreased with increases of the mechanical pressure applied. Except for the pressure range lower than 50 g., the decreases is found to be approximately linear with the pressure. At the highest pressure, 300 g. in these cases, the electrical excitability is about 60 per cent of the resting level. The fact that under the three different conditions similar results were obtained suggests that the effect of pressure remains unchanged for at least 3 seconds after removal of the pressure. It is to be noted that this seems to hold good for the measurement carried out with pressure as low as 50 g.

2. Dependence of electrical excitability of the eye on the duration

of pressure

In the next series of experiments, the dependence of excitability on the duration of pressure was investigated.

The results are shown in Fig. 3, in which percentage excitability is plotted against duration of pressure in seconds. In these experiments the electrical test shock was applied 0.5 seconds after removal of pressure,

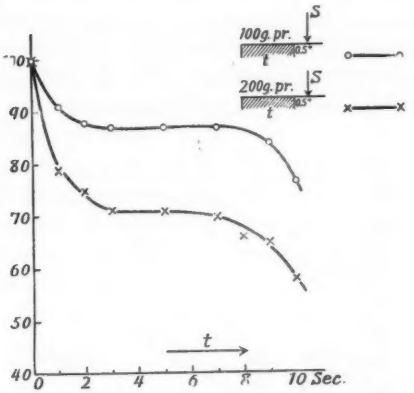

Fig. 3. Relation between duration, t, of pressure and electrical excitability of eye.

because the effect of pressure is known to remain unchanged for a few seconds as has been shown above. The upper and the lower curves refer to pressure values of 100 and 200 g. respectively. The excitability decreases as the duration of pressure increases, but no further decrease occurs between 3 and 7 seconds. At longer duration the decrease becomes remarkable again. This relation may be considered to represent the time course of the effect of a certain constant pressure applied to the eyeball, although each point of the course has, in reality, been determined in repeated sequences. Thus, three stages can be distinguished, that is, an initial stage of rapid change, an intermediate stage of steady state, and a final stage of the second rapid change.

These three stages could be found with any intensity of pressure so far used. From comparison of the two curves for pressure values, 100 and 200 g., it may be said that the intensity of pressure is more effective to reduce the electrical excitability of the eye than is the duration.

3. Recovery from the effect of pressure

Recovery of electrical excitability after removal of pressure was investigated.

The period of pressure application was varied from 1 to 10 seconds,

two intensities 100 and 200 g. being used. The results are shown in Fig. 4, in which open circles and crosses refer to the data obtained with 100 and 200 g. respectively.

From the curves shown in Fig. 4 the following general features can be found: 1) The longer the duration of pressure the longer the recovery time; for instance, recovery times are 5, 7, 8 and 10 seconds when pressure of 100 g. lasted for 1, 3, 6 and 10 seconds respectively.

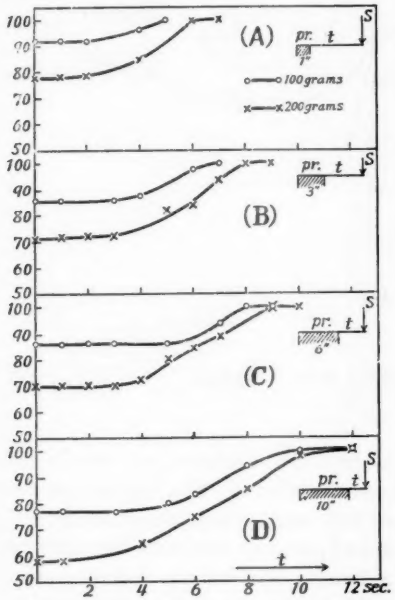

Fig. 4. Recovery from pressure effect. Ordinates: electrical excitability in percentage of resting level. Abscissae: time in seconds from termination of pressure.

2) The time course of recovery is S-shaped; at the initial stage, recovery is very slow or no recovery occurs. This stage lasts for 2 or 3 seconds. At the intermediate stage, recovery is more rapid, but becomes slower again at the final stage.

3) The recovery time is longer for 200 g. than for 100 g., but the difference is only small. It is to be noted that a brief mechanical pressure of 100 g. lasting only 1 second causes an after-effect of such long duration as 4–5 seconds.

4. Pressure and retinal circulation

In all the above-mentioned experiments the eye-disc of the form

illustrated in Fig. 2 was used to transmit pressure to the eyeball, and the pressure was expressed in grams.

The question arose as to whether the effect of mechanical pressure should depend on the size of the circular opening in the eye-disc. This question was important, because the opening had to be widened for ophthalmoscopic examination of the retina subjected to the pressure. In the following experiments three kinds of eye-disc were used; one was a disc 25 mm. in diameter without any opening, the second a disc of the same size with an opening 6 mm. in diameter, and the third a similar disc with an opening three times as great as the former. The pressure was applied for 3 seconds and the electrical excitability was tested 3 seconds after removal of the pressure.

The data obtained are shown in Fig. 5, in which percentage excitability is plotted against pressure. The data obtained with the three different eye-discs coincided satisfactorily with one another in two subjects whose data are represented by the upper and the lower diagrams of Fig. 5. This fact indicates that the effect is independent of the size of the

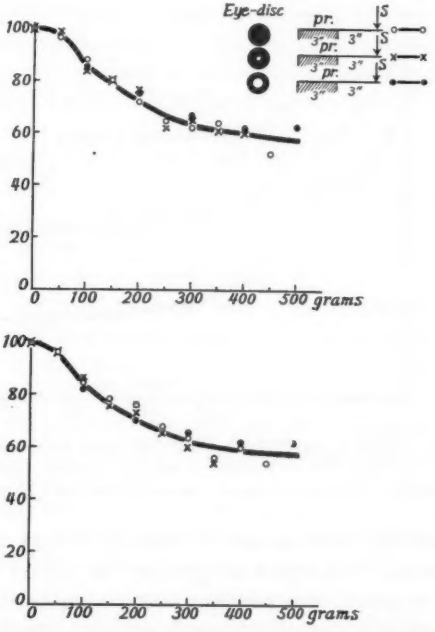

Fig. 5. Experiments showing that pressure effects are independent of the size of the circular opening in the eye-disc. Upper and lower curves were obtained different individuals.

opening in the eye-disc. After confirmation of this situation I tried to determine the value of pressure required to check the pulsation of the central artery of the retina, using the eye-disc of a wider opening and an ophthalmoscope.

The examination was carried out in a subject lying on a bed. The eye-disc mentioned above was held in place in the same manner as in the experiments on electrical excitability. This time, however, weights for a balance were used instead of a spring to apply pressure to the eye-disc. In three subjects it was found that retinal circulation was just checked by a pressure of 460–480 g.

5. Influence of pressure on the photosensitivity of the retina

In Fig. 6, dark-adaptation curves obtained after preliminary light adaptation of 5 minutes to an illumination of 1000 millilamberts are illustrated. They were determined with Nagel's adaptometer. A target of 10° in visual angle was used. A pressure apparatus of greater size had to be used, for the visual field of the apparatus employed above was too narrow to cover an area of 10° in visual angle. The larger one illustrated in Fig. 1 proved to be useful for this purpose.

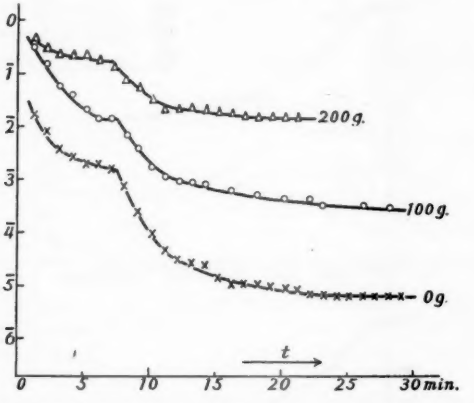

Fig. 6. Effect of compression of eyeball upon dark-adaptation curves. Intensity of pressure for each curve is given in grams. Ordinates: log of light thresholds. Abscissae: time in minutes from onset of dark-adaptation.

Light thresholds were measured from 3 to 8 seconds after the onset of pressure, because the effect of pressure is steady during this period (cf. Fig. 3). As usual, two portions can clearly be distinguished in the normal adaptation curve determined without application of pressure (the bottom curve in Fig. 6).

Light thresholds are generally increased by mechanical pressure

(cf. curves labeled 100 and 200 g. with bottom curve). It seems that increases of thresholds in the initial portion or the so-called cone-branch are slighter than those in the later portion or the rod-branch. No qualitative change, however, occurs in the adaptation curve, Kohlrausch's kink appearing at about the same time from the onset of dark-adaptation irrespective of the magnitude of pressure applied.

Discussion

In a previous paper, Motokawa *et al.*[8] reported that the electrical excitability of the eye rapidly decreased under low atmospheric pressure, but recovered promptly on oxygen supply. Hecht *et al.*[9] and McFarland and others[10] reported also that visual function decreased on breathing air of low oxygen tension and recovered as soon as oxygen was supplied.

From these experiments, however, it is impossible to decide the question as to whether or not the observed impairment of electrical excitability or visual function was caused by local anoxia. In the present experiments local anoxia was produced by mechanical pressure applied to the eyeball.

Although the intensities of pressure applied were always lower than the critical value of pressure required to check retinal circulation, the decrease in electrical excitability of the eye caused by the pressure was conspicuous, and the magnitude of decrease ran parallel to the intensity of pressure. This fact indicates that the electrical excitability is determined by the retina itself, but not by the central nervous system. In the present experiments the electrical excitability did not decrease below 50% of the resting level even when a pressure near the critical one was applied for a few seconds. If such a pressure had been applied for a longer time, a further decrease would have been observed. However, no such experiment was done for fear of retinal injury.

At first it was surmised that the intact eye might have been stimulated by current spread and served to keep the electrical excitability above 50% of the resting level. But this supposition was not shown to be true, for each subject reported that electrical phosphenes appeared always in the eye subjected to pressure, but not in the contralateral intact one. Further it was confirmed that no further decrease was caused by compression of both eyeballs together.

The findings that both cone- and rod-branches of the dark-adaptation curve were affected by compression of the eyeball, and that effects were a little greater upon the rod-branch than upon the cone-branch seem to agree with the data obtained by McFarland and by others in their experiments carried out in pressure chambers. McFarland suggested that the changes were not concerned with the photochemical substances of the retina, but with the neural elements of both retina and central

nervous system.

As it is obvious that in my experiments, anoxia is confined to the eye, the central nervous system can not be involved. On the other hand there are a number of facts suggesting that the neural elements of the retina are a determining factor for the electrical excitability of the eye.[2),11)]

The present experiment has provided the most convincing evidence that the electrical excitability of the eye is determined by the physiological state of the retina itself.

Summary

1. In this experiments a special apparatus to compress the eyeball was devised, and effects of pressure on the electrical excitability of the dark-adapted eye were investigated.

2. The intensity of the electrical test stimulus, a rectangular pulse of 0.1 second was increased step by step from a subthreshold level, and threshold voltages necessary to evoke the minimal electrical phosphene were determined.

The electrical excitability or reciprocal of threshold was expressed in percentage of the resting level.

3. When pressure of 100–400 g. was applied to the eyeball, the electrical excitability decreased rapidly within a few seconds, and then remained constant for about 4–5 seconds, to be followed by a second rapid decrease. The steady level mentioned above remained always above 50% of the resting level. There was a linear relation between the intensity of pressure and the decrease of excitability caused by the pressure.

4. The effect of pressure of 1–10 seconds in duration and 100–200 g. in intensity remained unchanged about 3 seconds after removal of the pressure, and it took 5–10 seconds for complete recovery.

5. With the same pressure apparatus the critical value of pressure required to stop retinal circulation was determined by means of an ophthalmoscope in three subjects, and values 460–480 g. were obtained.

6. In order to investigate the effect of pressure on the photosensitivity, dark-adaptation curves were determined under pressure by Nagel's adaptometer, and it was found that light thresholds were increased by pressure in both cone- and rod-branches of dark-adaptation curves.

All these findings have provided evidence that the electrical excitability of the eye is determined by the state of the retina.

The writer is greatly indebted to Prof. K. Motokawa, under whose guidance this investigation was performed, for his generous advice and valuable suggestions throughout the course of the experiment and the preparation of the manuscript.

References

1) Bourguignon, G. & Dejean, C. R. Soc. Biol., 1926, **94**, 750.
2) Motokawa, K., J. Neurophysiol., 1949, **12**, 291.
3) Motokawa, K., *ibid.*, 1949, **12**, 465.
4) Motokawa, K., *ibid.*, 1949, **12**, 475.
5) Motokawa, K. & Suzuki, K., Tohoku J. Exp. Med., 1950, **52**, 341.
6) Motokawa, K., *ibid.*, 1951, **54**, 79.
7) Motokawa, K. & Isobe, K., J. Opt. Soc. Amer., 1955, **45**, 79.
8) Motokawa, K. & Iwama, K., Tohoku J. Exp. Med., 1949, **50**, 319.
9) Hecht, S., Hendly, C. D., Frank, S. R. & Haig, C., J. Gen. Physiol., 1946, **29**, 335.
10) McFarland, R. & Evans, J. N., Amer. J. Physiol., 1939, **127**, 37.
11) Granit, R., J. Neurophysiol., 1948, **11**, 253.

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Susceptibility of Newborn Mice of Resistant Strain to Chemical Cancerogen

By

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Newborn mice of both sexes, 1 to 7 days old, of dd-Strain were administered 1% suspension of 20-methylcholanthrene in 25% PVP[1] intraperitoneally. They were breast-fed by non-treated mother, and after weaning fed boild whole wheat, boiled and dried baby-sardine and green vegetables. Temperature of the animal quarter was regulated between 18° and 25°C. Incidence of lung tumor among thus treated mice was very high at the age of 3 months exceeding 75%. Whereas, among the untreated controls and mice administered the identical agent or subcutaneous injection of methylcholanthrene-rosin pellet[2] 30 days after birth the incidence was very low and retarded, not exceeding 1% and being detected after 8 months. Numbers of animals examined so far are too small to determine exact percentages and time of onset of tumor formation at this time. Tumors observed were histologically identical in all groups; that is, benign papillary adenocarcinoma,[3] presumably of alveolar origin. However, a tendency was noticed in the group treated at 30 days to form a single tumor mass at the caudal pole of the inferior lobe of the right lung accompanying minute scattered lesions in the whole lung. Tumor masses in the former group were small and numerous being diffusely disseminated in the entire lung. A distinct contrast between these two groups was noticed in regard to the local tumor formation; it frequently occurred at the site of introduction of the agent in the latter group, manifesting invariably as fibrosarcoma containing multi-nucleated giant cells. While in the former group very few mice developed fibrosarcoma, some of which are probably attributable to accidental inoculation of the agent. Such difference in the mode of tumor incidence doesn't seem to explain well the enhanced susceptibility of newborn mice to methylcholanthrene. Analysis of the mechanism of lung-tumor induction by administration of chemical cancerogens to newborn mice of ordinarily resistant strain is being undertaken and will be reported else where.

Reference: 1) Polyvinylpyrrolidone (Plasdone), provided by courtesy of General Aniline and Film Corporation, New York. 2) Imai, Acta Anatomica Japonica 1956, 31 Suppl. p. 7. 3) Cloudman, Biology of the laboratory mouse by the Staff of the Roscoe B. Jackson Memorial Laboratory, Dover, New York, 1941, pp. 210-212.

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Synthesis of An Artificial Group O Substance*

By

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In a foregoing work,[2] we synthesized various N-glycosides of N-acetylglucosamine-4-β-galactoside using phosphatidylpeptides, sphingosine and 1-O-palmitylsphingosine for aglycons, and found that most of the N-glycosides of phosphatidylpeptides were specifically B potent and those of sphingosine or 1-O-palmitylsphingosine specifically A potent. Furthermore it was noticed that one of the Group A active compounds showed group conversion from A to B with simultaneous mutarotation when stood as a neutral solution at 15°C, whereas one of the Group B compounds remained unaltered in either respect in a similar experiment. These findings suggested that the principal structural difference between a pair of natural Group A and B substances, obtainable from the same source and belonging to the same chemical category, is whether the determinant carbohydrate group is attached to the rest of the molecule α-glycosidically or β-glycosidically, and that the bare surface of the determinant group, of which the other surface is laterally attached to the aglycon, plays the role of adhering to α- or β-agglutinin.

Since then, we have made many attempts to prepare Group O substances by combining the disaccharide above with various kinds of compound such as α,β-dipalmitylglycerophosphoryl-ethanolamine,[3] α,α'-dipalmitylglycerophosphoryl-ethanolamine[4] and so on as aglycons, but all the compounds given were not Group O active. Hence, next, N-acetylgalactosamine-4-β-galactoside, glucose-6-β-galactoside and galactose-4-β-galactoside were synthesized, and these disaccharides and melibiose were attached N-glycosidically to sphingosine, dihydrosphingosine or α- or γ-glutamyl-(α,α'-dipalmitylglycerophosphoryl)-ethanolamine. The results of the hemagglutination inhibition assay of the N-glycosides obtained

* A part was read before the 27th Annual Meeting of the Japanese Biochemical Society, Kyoto, April 2-5, 1955.[1] 193rd report of "Biochemical Studies on Carbohydrates"; 24th report of "Chemistry and Biology of Lipids"; 11th report of "On Proteins and Amino Acids".

are embodied in Table I.

The derivatives of N-acetylgalactosamine-4-β-galactoside (**I**, **II** and **III**) and sphingosine-N-glucosido-6-β-galactoside (**IV**) inhibited the agglutination of all A, B and O cells by antisera, the degree of the inhibition varying from compound to compound and according to the group of cells. Sphingosine-N-galactosido-4-β-galactoside (**V**) proved to have no potency of inhibiting the agglutination of any group of red cells. Sphingosine-N-melibioside (**VI**) was specifically Group O active, while γ-glutamyl-(α,α'-dipalmitylglycerophosphoryl)-ethanolamine-N-melibioside (**VII**) showed some specific Group B activity.

Taken in conjuction with the findings in the foregoing article,[2] it may be presumed that 1 : There are at least two kinds of disaccharide, of which one including N-acetylglucosamine-4-β-galactoside can become the determinant group of both A and B substances and the other, to which N-acetylgalactosamine-4-β-galactoside and glucose-6-β-galactoside belong, can constitute that of all Group A, B and O substances. Regarding the latter kind of disaccharide we incline to believe that the sugar bound to such amphipatic compounds as used above exhibits O-activity, when it is held in suspended state, that is, neither of its both surfaces clings to the aglycon, and hence the greater the part of the bound sugar in that state is, the more Group O active is the conjugated substance. **I** is Group O active against anti-O saliva chicken serum but not against normal anti-O goat serum, and **II** is reversed in this connection. This fact may be explained by assumption of different deformations of the disaccharide molecule in them due to peculiar attraction and repulsion of the aglycon with which it is linked. Both **VI** which is Group O active and **VII** which is Group B active contain melibiose. This disaccharide would form a Group A substance, if it is combined with an aglycon which attracts and appropriately shields the surface of the carbohydrate opposite to the attached surface of the carbohydrate in **VII**. Melibiose is rather inclined to take the suspended form in its N-glycosides, since it has neither amino- nor acetamino-group.

Watkins and Morgan[5] pointed out the inhibitory effect of lactose, melibiose, galactose, and α- and β-methylgalactopyranoside to the action of the enzyme of Trichomonas foetus on B substance. According to Kabat and Leskowitz,[6] melibiose, raffinose and stachyose are excellent inhibitors of B-anti-B precipitation, and raffinose, melibiose and galactose inhibit the agglutination of B cells by anti-B. These authors announced therefrom that " a terminal non-reducing 1→6-α-galactopyranoside linkage is an important determinant of blood group B specificity." However, in our hemagglutination inhibition assays, in which normal antisera were used, those mono- and oligosaccharides showed no Group B activity at

all (Tables I and II).

TABLE I

Test for Inhibition of the Synthetical N-Glycosides, Melibiose and Stachyose to Agglutination of A, B and O Cells by Antisera, as Assayed by the Agglutinogen Dilution Method

I: sphingosine-N-(N-acetyl)-galactosaminido-4-β-galactoside, **II**: dihydrosphingosine-N-(-N-acetyl)-galactosaminido-4-β-galactoside, **III**: α-glutamyl-(α,α′-dipalmitylglycerophosphoryl)-ethanolamine-N-(N-acetyl)-galactosaminido-4-β-galactoside, **IV**: sphingosine-N-glucosido-6-β-galactoside, **V**: sphingosine-N-galactosido-4-β-galactoside, **VI**: sphingosine-N-melibioside, **VII**: γ-glutamyl-(α, α′-dipalmitylglycerophosphoryl)-ethanolamine-N-melibioside, **VIII**: melibiose, IX: stachyose. G: a normal anti-O goat serum, Anti-OS: an anti-OS saliva chicken serum. −: no agglutination, ±: faint aggl., +: slight aggl., ++: marked aggl., +++: strong aggl.

Compound	Serum	Red cells	Dilution of the substances (1:)							
			5× 10	10^2	2× 10^2	4× 10^2	8× 10^2	1.6× 10^3	3.2× 10^3	6.4× 10^3	∞
I	α	A		−	−	−	−	+	++	++	+++
	β	B		−	−	−	−	−	+	++	+++
	G	O		+++	+++	+++	+++	+++	+++	+++	+++
	Anti-OS	O		−	−	−	−	−	−	+	+++
II	α	A		−	−	−	−	+	++		+++
	β	B		−	−	−	−	−	+		+++
	G	O		−	−	−	±	+	++		+++
	Anti-OS	O		+	+	+	+	++	++		+++
III	α	A		−	−	±	+	+	++	++	++
	β	B		−	−	±	±	+	++	++	++
	G	O		−	−	−	−	−	±	+	+++
	Anti-OS	O		−	−	−	−	−	−	+	+++
IV	α	A		−	−	±	+	+	++	+++	+++
	β	B		−	−	±	+	+	++	+++	+++
	G	O		−	±	±	+	+	++	++	++
	Anti-OS	O		−	−	−	+	+	++	+++	+++
V	α	A		+	++	++	++	++	++		+++
	β	B		+	+	++	++	++	++		+++
	G	O		+	+	++	++	++	++		+++
	Anti-OS	O		++	++	++	++	++	++		+++
VI	α	A		++	++	++	++	++	++	++	++
	β	B		++	++	++	++	++	++	++	++
	G	O		−	−	−	±	+	+	+	++
	Anti-OS	O		−	−	−	−	−	−	±	++
VII	α	A		±	+	+	++	++	++	++	+++
	β	B		−	−	−	±	+	+	++	+++
	G	O		±	±	+	+	+	+	++	++
	Anti-OS	O		±	±	+	++	++	++	++	++
VIII	α	A	+	++	++	+++	+++	+++	+++		+++
	β	B	+	++	++	+++	+++	+++	+++		+++
	G	O	+	+	++	+++	+++	+++	+++		+++
	Anti-OS	O	+	+	++	++	+++	+++	+++		+++
IX	α	A	+	++	++	+++	+++	+++	+++		+++
	β	B	+	++	++	++	++	+++	+++		+++
	G	O	++	++	++	++	++	+++	+++		+++
	Anti-OS	O	+	++	++	++	++	+++	+++		+++

TABLE II

Test for Inhibition to Agglutination of B Erythrocytes by Normal Anti-B Serum

Kabat and Leskowitz's antibody dilution method[6] was applied. Sugars added were each 2 mg. Symbols −∼## signify the same as in Table I.

Sugar tested	Dilution of the anti-B serum (1:)									
	2.5	5	10	2× 10	4× 10	8× 10	1.6× 10^2	3.2× 10^2	6.4× 10^2	1.28× 10^3
None	##	##	##	##	##	+	+	±	±	−
Galactose	##	##	##	##	##	+	+	±	±	−
Lactose	##	##	##	##	##	+	+	+	−	−
Melibiose	##	##	##	##	##	+	+	±	−	−
Raffinose	##	##	##	##	##	+	+	+	±	−

EXPERIMENTAL

Synthesis of the N-Glycosides

N-Acetylgalactosamine-4-β-galactoside

Preparation of N-acetylgalactosamine. 10 g. of crystalline galactosamine hydrochloride isolated according to Hiyama[7] were N-acetylated after Roseman and Ludowieg.[8] Yield 9.2 g. The product softened at 125° and melted at 162–165°.

Preparation of 6-trityl-N-acetylgalactosamine. N-acetylgalactosamine was tritylated with tritylchloride[9] in dry pyridine according to Helferich *et al.*[10,11] and separated from tritanol chromatographically by following Zeile and Kruckenberg,[12] as follows. 10 g. of N-acetylgalactosamine and 16 g. of tritylchloride were mixed well, dissolved in 240 cc. of abs. pyridine and stood at room temperature (20–23°) for two days with occasional shaking. The reaction mixture was subsequently distilled in vacuo and the still-residue was kneaded with changes of water, until it became fluffy, and spread on a porous plate. About 20 g. of a yellowish white powder were obtained. Next it was exhausted with petrol ether to remove tritanol. The remainder was dissolved in 150 cc. of dry benzene (thiophene free). The solution was poured onto a 3×25 cm. column of alumina powder LBT (Nippon Aluminum Co.), packed with benzene under suction and chromatographed with 400 cc. of benzene whereby the remaining tritanol was eluted. Subsequent elution was effected with 500 cc. of ethanol, and the effluent here was vacuum-distilled to a thick solution and placed in a refrigerator. Fine needles came out in bundles. Yield 4.8 g. The crystals sintered at 131–132° and melted at 140° under foaming. Recrystallization did not elevate the melting point. N (micro Kjeldahl) 3.05% (Calcd. for $C_{27}H_{29}O_6$ 3.02%).

Preparation of aniline-N-(6-trityl-N-acetyl)-galactosaminide. 2.34 g. of 6-trityl-N-acetylgalactosamine, 0.59 cc. of freshly distilled aniline and 0.1 cc. of glacial acetic acid were dissolved together in 15 cc. of abs. methanol and refluxed at

80° in an oil-bath for 3 hours, followed by vacuum distillation to dryness. The still-residue was taken up in 4 cc. of methanol and after addition of dry ether until slight cloudiness, stood in an ice-chest. Since a mere oily deposit occurred thereby, it was dissolved by increasing methanol, and the solution was added to with petrol ether of low boiling point until persistent turbidity, and stood in an ice-chest for three days. The amorphous precipitate turned gradually into a conglomerate of fine needles. It was filtered, washed with a mixture of methanol, ether and petrol ether (1:1:1 by volume) and dried in vacuo. A white crystalline powder given amounted to 1.65 g. It softened at 58° and melted at 62°. N (micro Kjeldahl) 5.08% (Calcd. for $C_{33}H_{34}O_5N_2$ 5.20%). Aniline[13] 16.5% (Calcd. 17.2%).

This compound turns yellowish and then brown even when kept in vacuo, so that the product was soon submitted to the following stage of preparation.

Preparation of aniline-N-(6-trityl-N-acetyl)-galactosaminido-4-β-galactoside. A modification of Helferich mercuric cyanide method[14] was applied. 1.6 g. of aniline-N-(6-trityl-N-acetyl)-galactosaminide, perfectly dried in vacuo at 56°, and 0.98 g. of acetobromogalactose were dissolved in 5 cc. of dry benzene and vigorously shaken with 0.724 g. of mercuric cyanide and 0.3 g. of a dry powder of ovalbumin, whereby the pH of the solution decreased quickly and became lower than 1.0 in 90 minutes. The mixture was neutralized with sodium acetate (anhydrous), further shaken for 1 hour and filtered through an active carbon layer on a hard filter paper. The bulk on the funnel was washed with benzene energetically, and the filtrate and washing were united and distilled under reduced pressure to syrupy consistency. The still-residue contained bromide scarcely and gave much weaker Fehling reaction than acetobromogalactose. It was dissolved in 3.0 cc. of dry pyridine, added to with 2.5 cc. of acetic anhydride, and after standing at 22–24° for 24 hours, poured into 100 cc. of iced water under agitation. After a few hours the supernatant was decanted out, and the oily deposit at the bottom of the vessel was repeatedly kneaded with water until it changed fluffy, filtered with suction and dried in air. Yield 1.8 g. It was next dissolved in abs. methanol, decolorized by boiling with active charcoal and filtered, followed by washing with methanol. The filtrate and washing were distilled in vacuo to dryness and dissolved in the smallest possible quantity of ethanol (3 cc.), and 2.5 cc. of petrol ether were added in drops. The faintly opalescent solution was stood overnight with occasional scratching of the vessel wall. And the crystalline deposit was filtered off. This looked to be penta-acetylgalactose because it contained only 0.08% N and melted at 145–147° under decomposition. The filtrate was distilled in vacuo, and the dry remainder was taken up in 5 cc. of a mixture of benzene and methanol (4:1 by volume) and passed through an alumina column of 1×10 cm. size. The column was then washed with 10 cc. of the same solvent mixture. The non-colored effluent and washing were distilled together in vacuo to dryness. Many trials to change the still-residue into crystals failed. Hence a thick methanolic solution of it was precipitated by means of a large excess of an ether-petrol ether mixture (1:1 by volume), centrifuged and dried in vacuo over H_2SO_4. The powder yielded weighed 1.4 g. N (micro Kjeldahl) 3.52% (Calcd. for $C_{49}H_{54}O_{15}N_2$

3.07%). Galactosamine (Masamune and Yosizawa)[15] 23.5% (Calcd. 19.6%). Namely, the substance proved to contain no little amount of the unaltered galactosaminide as a contamination.

Elimination of trityl, acetyl and aniline residues. Detritylation was made after Kuhn *et al.*[16] and deacetylation with alkali. 1.3 g. of the substance above were dissolved in 20 cc. of 80% acetic acid and refluxed in a boiling water-bath for 30 minutes, whereby the reaction mixture got colored brown. Aniline was also cleft off by this treatment. The solution was cooled in water thereupon and distilled in vacuo to about 1/3 the volume. It was freed from crystalline tritanol that had come out, and after exhaustion with petroleum benzin several times, distilled at a low temperature to a syrup, and the acetic acid was expelled off by redistillations of the still-residue with water. The dirty thick solution after the final distillation was neutralized with 10 cc. of 0.4 *N* NaOH and shaken with 50 cc. of 0.2 *N* NaOH for 9 hours. The substance once precipitated dissolved slowly. The dark brown insoluble part was filtered off, and the filtrate was shaken in a separatory funnel with petroleum benzin to remove aniline. The underlayer that separated after standing was agitated with 20 cc. portions of Amberlite IR-120 and IR-4B and distilled in vacuo to a syrup. This was proved paper-chromatographically to contain acetylgalactosamine and a trace of galactose besides the disaccharide in view, which was isolated by charcoal-chromatography of Whistler and Durso,[17] as follows. Equal amounts of charcoal, purified and activated, and Celite were packed into a 20 cm. high column in a 2.5×40 cm. chromatographic tube and washed with water. The syrup above was dissolved in 10 cc. of water, poured onto the column and washed afterwards with 600 cc. of water until no more Fehling-positive substance flowed out. The substance remaining absorbed was eluted with about 800 cc. of 8% ethanol. The eluate was distilled in vacuo to a thick solution, transferred to a small test tube and placed in a vacuum desiccator. The evaporated residue was taken up in as little methanol as possible (2 cc.), and after addition of 3 cc. of ethanol, stood in a refrigerator for two days with occasional scratching of the vessel wall. Crystals appeared gradually. The deposit was centrifuged, washed with chilled abs. ethanol and dried in vacuo over $CaCl_2$. Yield 60 mg. It contracted at 139° and melted at 145°. $[\alpha]_D^{20}$ in water was $\frac{0.927 \times 100}{1 \times 1.12} = +82.5°$ at the fifth minute after dissolution and $\frac{0.778 \times 100}{1 \times 1.12} = +69.3°$ at equilibrium (after 5 hours from dissolution). N 3.46%, iodine use (Macleod and Robison) as glucose 44.8%, galactosamine (Masamune and Yosizawa[15]) 46.9%. Calcd. for $C_{14}H_{25}O_{11}N$, N 3.65%, iodine use as glucose 46.9%, galactosamine 46.7%.

The procedure above was repeated to obtain a sufficient amount of this substance.

It was proved that galactose was attached to C atom 4 of N-acetylgalactosamine glycosidically, because the disaccharide gave only faintly positive indirect-Osaki-Turumi reaction, and consumed approximately 3 moles of periodate per mole, as will be detailed:—39.3 mg. (1×10^{-4} mole) of the disaccharide and

36.0 mg. (1×10^{-4} mole) of lactose monohydrate were weighed each in a test tube, added to with 1 cc. of water and 1 cc. of 0.5029 *M* sodium metaperiodate[18] in turn and stood in a water-bath at 20° for 100 minutes. Periodate remaining unreduced was estimated by an arsenite method.[19] It amounted to 0.2047 and 0.0317 mole in the respective cases, in other words, the disaccharide in question and lactose consumed 2.982 ($=\frac{(0.5029-0.2047)\times10^{-3}}{1\times10^{-4}}$) and 4.712 ($=\frac{(0.5029-0.0317)\times10^{-3}}{1\times10^{-4}}$) mole of periodate per mole respectively.

Sphingosine-N-(N-acetyl)-galactosaminido-4-β-galactoside (**I**)

40 mg. of free sphingosine freshly prepared (from Carter's sphingosine sulfate Fr. II[20]) and 60 mg. of N-acetylgalactosamine-4-β-galactoside were boiled with 3 cc. of abs. ethanol at 90° (glycerol-bath) for 5 hours in a current of dry CO_2 gas under a return condensor fitted with a $CaCl_2$ tubelet. When cold, the reaction mixture was distilled in vacuo to a syrup and taken up in 5 cc. of dry chloroform. The insoluble part was rejected after centrifugation. The solution was concentrated to a thick solution as above, precipitated with a large excess of dry acetone and washed with the same solvent. It was then dissolved in 1 cc. of abs. methanol, and after addition of dry ether in drops until slight opalescence, placed in a refrigerator overnight. A white deposit in granules was centrifuged off and dried in vacuo over $CaCl_2$. Yield 25.5 mg. It sintered at 119° and melted at 121°. N (micro Kjeldahl) 4.12% (Calcd. for $C_{32}H_{59}O_{12}N_2$ 4.22%), galactosamine (Masamune and Yosizawa[15]) 25.3% (Calcd. 26.9%). It was soluble in chloroform, ethanol, methanol, wet ether and water, but insoluble in ether (dry), petrol ether and acetone.

Dihydrosphingosine-N-(N-acetyl)-galactosaminido-4-β-galactoside (**II**)

300 mg. of Carter's Fr. I of sphingosine sulfate[20] were liberated from sulfuric acid, dissolved in a mixture of 50 cc. of ethanol and 8 cc. of glacial acetic acid and hydrogenated in the presence of 0.1 g. of palladium black. 150 cc. of hydrogen were consumed. The yield of dihydrosphingosine was 290 mg. F. P. 105–109°. 30 mg. of it were condensed with 50 mg. of N-acetylgalactosamine-4-β-galactoside similar to above, and 18 mg. of the N-glycoside were given. F. P. 125–128°. N (micro Kjeldahl) 4.08% (Calcd. for $C_{32}H_{61}O_{12}N_2$ 4.19%).

α-Glutamyl-(α,α'-dipalmitylglycerophosphoryl)-ethanolamine-N-(N-acetyl)-galactosaminido-4-β-galactoside (**III**)

α-Glutamyl-(α,α'-dipalmitylglycerophosphoryl)-ethanolamine was prepared as described in the foregoing article[2]. F. P. 52°. N 2.7%, P 3.25%. 150 mg. of it were mixed with 60 mg. of N-acetylgalactosamine-4-β-galactoside and boiled with 4 cc. of a 1:1 mixture of equal volumes of abs. methanol and abs. ethanol under reflux for 12 hours, avoiding moisture. When cold, the reaction mixture was distilled in vacuo to a syrup, dissolved in 8 cc. of dry ether by slight heating and chilled in ice-water. The occurring deposit[21] was rejected after centrifugation. The supernatant was distilled under reduced pressure to dryness and further dried in a vacuum desiccator containing H_2SO_4. It was then dissolved by warming in 5 cc. of a dry ether-petrol ether mixture in the

volume proportion of 1:1 and cooled in ice-water. An appearing voluminous precipitate was centrifuged off and after washing exhaustively with the ether-petrol ether mixture ice-cooled, dried in vacuo over $CaCl_2$. 28 mg. of a fluffy powder were given. It contracted with slight browning at 96° and decomposed at 167°. Molisch strongly positive. It gave an opalescent emulsion in water, and was soluble easily in methanol, chloroform, hot ethanol and hot ether, but difficultly in cold ethanol, cold ether and acetone. N (micro Kjeldahl) 3.42%, P (a modification of Plimmer method[22]) 2.78%, galactosamine (Masamune and Yosizawa[15]) 13.5%. Calcd. for $C_{55}H_{104}O_{21}NP$, N 3.58%, P 2.81%, galactosamine 15.26%. $[\alpha]_D^{25}$ in an ethanol-chloroform mixture (1:1 by volume) was $\frac{0.198\times100}{1\times1.25}=+15.8°$ at the fifth minute after dissolution and $\frac{0.181\times100}{1\times1.25}=+14.5°$ at equilibrium (after 10 hours from dissolution).

Sphingosine-N-glucosido-6-β-galactoside (**IV**)

50 mg. of glucose-6-β-galactoside, which had been prepared as will be reported in an article from this Institute,[23] and the same amount of a fresh preparation of free sphingosine (from Carter's sphingosine sulfate Fr. II) were boiled with 3 cc. of abs. ethanol for 5 hours under reflux, keeping off moisture, and when cold, the reaction mixture was distilled in vacuo to dryness. The still-residue was taken up in 5 cc. of chloroform and stood in a refrigerator. The occurring deposit was centrifuged off and the supernatant was distilled as above. After further drying in vacuo over $CaCl_2$, it was washed with dry ether to eliminate the part of sphingosine that had not reacted, and again dried. A white powder obtained was dissolved in the smallest possible quantity of abs. ethanol, carefully added to with dry ether until faint cloudiness and stood in a refrigerator. The voluminous precipitate was centrifuged off. Trials to change it into crystals failed so that it was dried as such in vacuo over $CaCl_2$. 35 mg. was the yield. It melted at 98–115°. N (micro Kjeldahl) 2.25% (Calcd. for $C_{30}H_{57}O_{12}N$ 2.24%). $[\alpha]_D^{15}$ in ethanol was $\frac{0.570\times100}{1\times1.62}=+35.2°$ at the 5th minute after dissolution and $\frac{0.364\times100}{1\times1.62}=+22.5°$ at equilibrium (after 10 hours from dissolution).

Sphingosine-N-galactosido-4-β-galactoside (**V**)

20 mg. of sphingosine (from Carter's sphingosine sulfate Fr. II) and 30 mg. of galactose-4-β-galactoside, which had been prepared as will be published elsewhere[23], were mixed and heated with 2 cc. of abs. methanol for 4 hours, avoiding moisture. The sugar went into solution soon after the commencement of boiling. The reaction mixture was decolorized with active charcoal and distilled in vacuo to complete dryness. The still-residue was taken up in 10 cc. of purified ether (not dried) and the insoluble part was rejected after centrifugation. The supernatant was distilled in vacuo to perfect dryness, washed with dry ether and dried in vacuo over $CaCl_2$. It was further precipitated from 1 cc. of ethanol with a large excess (10 cc.) of dry acetone. 25 mg. was the yield. The product contracted at 110° and melted at 135–138° under

browning. Its iodine use (Macleod and Robison) after hydrolysis[24] amounted to 57.5% as expressed as glucose (Calcd. for $C_{30}H_{57}O_{11}N$ 59.6%). N (micro Kjeldahl) 2.25% (Calcd. 2.30%). $[\alpha]_D^{22}$ in abs. ethanol was $\frac{0.280\times100}{1\times1.520}=+18.5°$ at the 10th minute from dissolution and $\frac{0.737\times100}{1\times1.520}=+48.5°$ at equilibrium (after 18 hours from dissolution).

Sphingosine-N-melibioside (**VI**)

50 mg. each of sphingosine (from Carter's sphingosine Fr. II) and melibiose were condensed with each other similar to above. The product, separated as above, came out in lustrous prisms from ethanol when stood after addition of ether. The crystals were separated and dried in vacuo over $CaCl_2$. Yield 32 mg. It softened at 160° and melted at 172–176° under decomposition. N (micro Kjeldahl) 2.12% (Calcd. for $C_{30}H_{57}O_{12}N$ 2.24%). $[\alpha]_D^{15}$ in ethanol was $\frac{0.720\times100}{1\times1.59}=+45.3°$ at the 5th minute after dissolution and $\frac{0.905\times100}{1\times1.59}=+56.9°$ at equilibrium (after 5 hours from dissolution).

γ-Glutamyl-(α,α'-dipalmitylglycerophosphoryl) -ethanolamine-N-melibioside (**VII**)

80 mg. of γ-glutamyl-(α,α'-dipalmitylglycerophosphoryl)-ethanolamine prepared in the manner described in the foregoing article[2] were mixed with 40 mg. of melibiose and boiled with 3 cc. of a 1:1 mixture by volume of abs. ethanol and abs. methanol for 12 hours under a return condensor fitted with a $CaCl_2$ tubelet. A precipitate came out on standing the reaction mixture in air. It was centrifuged and washed with ethanol. The supernatant and washings were distilled in vacuo to complete dryness, and the still-residue was extracted with chloroform. The chloroformic solution was clarified by filtration and distilled again to complete dryness. The still-residue here was taken up in 6 cc. of a hot ether-ethanol mixture (3:1 by volume) and allowed to cool. A crystal-like deposit that appeared was centrifuged, washed with the ether-ethanol mixture and dried in vacuo over $CaCl_2$. Yield 25 mg. It softened with browning at 155° and decomposed at 167°. Its iodine use (Macleod and Robison) after hydrolysis[24] amounted to 30.3% as glucose (Calcd. for $C_{51}H_{101}O_{21}N_2P$ 31.4%). N (micro Kjeldahl) 2.35% (Calcd. 2.44%).

Hemagglutination Inhibition Test

The assay was processed on a glass plate as described in the foregoing article[2], excepting when galactose, lactose, melibiose and raffinose were examined by progressive dilution of a normal anti-B serum. The results are embodied in Tables I and II.

Summary

1. Sphingosine-N-(N-acetyl)-galactosaminido-4-β-galactoside (**I**), dihydrosphingosine-N-(N-acetyl)-galactosaminido-4-β-galactoside (**II**), α-

glutamyl-(α, α'-dipalmitylglycerophosphoryl)-ethanolamine-N-(N-acetyl)-galactosaminido-4-β-galactoside (**III**), sphingosine-N-glucosido-6-β-galactoside (**IV**), sphingosine-N-galactosido-4-β-galactoside (**V**), sphingosine-N-melibioside (**VI**) and γ-glutamyl-(α,α'-dipalmitylglycerophosphoryl)-ethanolamine-N-melibioside (**VII**) were synthesized and examined regarding their potencies inhibiting hemagglutination of A, B and O erythrocytes by antisera.

2. **I–IV** are inhibitory to the agglutination of all A, B and O erythrocytes although the inhibition degree varies from compound to compound and from antiserum to antiserum. **V** is not active at all. **VI** is specifically Group O(H) active, whereas **VII** which contains melibiose like **VI** is specifically Group B active, though weak. The disaccharides employed for the synthesis of the N-glycosides in 1., galactose, melibiose, lactose, raffinose and stachyose are devoid of any of those inhibitory powers.

3. Based on the findings, the spatial configuration of O substances is discussed.

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References and Notes

1) Masamune, H., Hakomori, Kamiyama & Masamune, O., Seikagaku (Transactions Jap. Bioch. Soc.), 1955, **27**, 414.

2) Masamune, Hakomori, Numabe, Akama & Kamiyama, Tohoku J. Exp. Med., 1955, **61**, 283.

3) Synthesized according to Baer, Maurukas & Russel (J. Am. Chem. Soc., 1952, **74**, 154).

4) Synthesized according to Rose, J. Am. Chem. Soc., 1947, **69**, 1384.

5) Watkins & Morgan, Nature, 1955, **175**, 676.

6) Kabat & Leskowitz, J. Am. Chem. Soc., 1955, **77**, 5159.

7) Hiyama, Tohoku J. Exp. Med., 1949, **50**, 379.

8) Roseman & Ludowieg, J. Am. Chem. Soc., 1954, **76**, 301.

9) Prepared by the improved method by Reynolds and Evans of Gomberg synthesis (J. Am. Chem. Soc., 1938, **60**, 2559).

10) Helferich, Speidel & Toeldte, Ber. dtsch. chem. Ges., 1923, **56**, 766.

11) Helferich, Moog & Jungers, *ibid.*, 1925, **58**, 872.

12) Zeile & Kruckenberg, Chem. Ber., 1942, **75**, 1127.

13) 6.82 mg. of the substance were hydrolyzed by heating with 3 cc. of 2 *N* HCl at 100°C for 30 minutes, and the hydrolysate was alkalinized with 1 *N* NaOH and extracted with changes of ether. The ethereal solutions were added to with 3 cc. of water, evaporated on a water-bath until all the ether was expelled off and transferred to a 10 cc. measuring flask, followed by filling up to the mark with water. 3 cc. portions were analyzed colorimetrically according to Tsuiki (Tohoku J. Exp. Med., 1955, **61**, 261).

14) Helferich & Wedemeyer, Liebig's Ann. Chem., 1948, **563**, 139.

15) Masamune & Yosizawa, Tohoku J. Exp. Med., 1956, **64**, 53.

16) Kuhn, Rudy & Weygand, Ber. dtsch. chem. Ges., 1936, **69**, 1546.
17) Whistler & Durso, J. Am. Chem. Soc., 1950, **72**, 677.
18) Jackson & Hudson, *ibid.*, 1937, **59**, 998; Jackson, Organic Reactions, John Wiley & Sons, New York, II, 1949, 341.
19) Fleury & Lange, J. pharm. et chim., 1933, **17**, 107.
20) Carter, Norris, Glick, Phillips & Harris, J. Biol. Chem., 1947, **170**, 274.
21) It was devoid of blood group activity despite of the strong Molisch reaction.
22) Haneda, Sinokawa & Yasuoka, Tohoku J. Exp. Med., 1944, **46**, 263.
23) Masamune & Kamiyama, *ibid.*, in press.
24) The substance was hydrolyzed by heating with 1 *N* H_2SO_4 at 100° for 4 hours.

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Biochemical Studies on Carbohydrates

CXCIV. An Improvement of the Fractionated Determination Method by Paper Partition Chromatography of Glucosamine and Galactosamine

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The writers[1] devised formerly a method for fractionated determination of the hexosamines. In that method, however, intensity of the color developed by the Ehrlich reagent differed between glucosamine and galactosamine, entailing some inaccuracy of analysis. Since then, Rondle and Morgan[2] informed that the both hexosamines are colored in equal strengths when solutions of the hexosamines are treated with half the volume of an acetylacetone solution in 0.5 *N* Na_2CO_3. The alkalinity of their reaction mixture was lower than that of the corresponding mixture in the writers' method. Therefore the Na_2CO_3 concentration of the acetylacetone reagent has been decreased to 0.7 normality after careful study. Secondly, the concentration of hydrochloric acid for hydrolysis of the unknown for total hexosamine analysis has been changed into 4 normality, after comparing hydrochloric acid solutions of various concentrations as to their effect on the succeeding color development in several mucopolysaccharides. Thirdly, Rondle and Morgan's technique was adopted at the stage of color development with Ehrlich reagent. Fourthly, the technique to locate the border between the spots of galactosamine and of glucosamine in the chromatogram has been altered so as to give a more reproducible result.

Goldschmiedt-positive mucopolysaccharides are difficultly hydrolyzed as compared with the Molisch-positive, and more or less of their unit disaccharide remains unhydrolyzed unless HCl of a higher concentration is used. The writers recommend hydrolysis with 6 *N* HCl for 15 hours for the chromatographic fractionation of the hexosamines for mixtures of Molisch-positive and Goldschmiedt-positive mucopolysaccharides and for those of Goldschmiedt-positive mucopolysaccharides, although, in this case, practically insignificant percentages of the hexosamines are unavoidably decomposed.

Analytical Techniques

Analysis of Total Hexosamines

1) A quantity of the unknown material containing 0.15–0.50 mg. in total of hexosamines is weighed in a well-stoppered (ground joint) glass tube, constricted and marked at 10 cc., and added to with 1 cc. of water and with 1 cc. of 8 *N* HCl in turn. The solution is heated for 4 hours in a boiling water-bath and cooled in air. After addition of one drop of 0.5% phenolphthalein in 50% alcohol, the hydrolysate is chilled in ice and neutralized with 1.5 cc. of 4 *N* NaOH and a requisite quantity of 0.5 *N* NaOH to distinct red, followed by immediate addition of 0.3 *N* HCl just till it becomes acidic to BTB. Then, it is filled up to the mark of 10 cc. with water (The concentration of NaCl in this solution 4.68%) and filtered when necessary.

2) 2 cc. of the solution thus prepared is pipetted into a glass tube with a stopper, wherein water is enclosed, (ground joint) (Fig. 1) and 1 cc. of an acetylacetone reagent (Dissolve 1 cc. of acetylacetone in 50 cc. of 0.7 *N* Na_2CO_3) is added. The tube is tightly stoppered, placed in a vigorously boiling water-bath for 20 minutes (The tube is beaten frequently to let the acetylacetone condensed at the stopper fall) and cooled in water. The reaction mixture is then diluted with 6 cc. of abs. ethanol.

3) To it 1 cc. of Rondle and Morgan's Ehrlich reagent (Dissolve 0.8 g. of *p*-dimethylaminobenzaldehyde in a mixture of 30 cc. of conc. HCl (sp. gr. 1.18) and 30 cc. of abs. alcohol) is added slowly, and the mixture is heated for 10 minutes in a water-bath maintained at 65–70°C (The tube is loosely stoppered) and cooled in water at 15–18°C to measure the extinction at the wave-length of 537 mμ against the blank.

A standard glucosamine hydrochloride or galactosamine hydrochloride solution in 4.68% NaCl is subjected to the operations 2) and 3) in parallel with the neutralized and diluted hydrolysate.

Calculation. The extinctions of the colors developed by equal quantities of glucosamine and galactosamine are the same and furthermore the extinctions for various amounts of the hexosamines obey Lambert-Beer law. Therefore, the total amount of the hexosamines can be calculated from the intensity ratio of the colors developed by the hydrolysate of the unknown and the standard hexosamine solution.

Fractionated Determination of the Hexosamines in Molisch-positive Mucopolysaccharides or Glucidamins

A) The total amount of the hexosamines is estimated as above.

B) The same solution of the unknown (a Molisch-positive muco-

polysaccharide or a glucidamin) that is subjected to the procedure in A) is hydrolyzed in a similar manner. The hydrolysate is freed from the hydrochloric acid as far as possible as described in the foregoing report[1] (It is advisable to place the absorbing agents over the hydrolysate in a vacuum desiccator for the purpose of quick evaporation). The water-insoluble part is removed halfway. The final evaporated residue is taken up in 0.05 or 0.1 cc. of water. Three equal portions of the solution are separately sampled at positions $\times$'s on a 15.5×56 cm. square of Toyo Roshi filter paper No. 3 shown in Fig. 2, and descending irrigation with

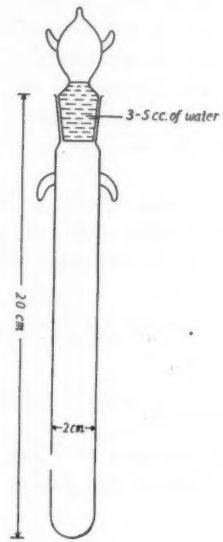

Fig. 1

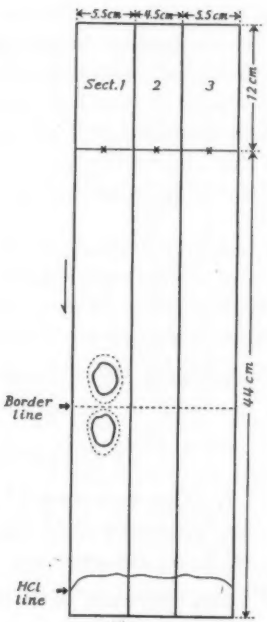

Fig. 2

the solvent mixture of butanol, pyridine and dilute hydrochloric acid is effected, as described also in the foregoing report.[1] When the pale yellow line falling of hydrochloric acid reaches near the bottom, the paper square is dried in air and washed by passing through dry ether. Sections 1–3 are cut asunder, and one of them is sprayed with the aniline hydrogen phthalate reagent of Partridge to locate the border between the two spots of the hexosamines. A straight horizontal line is pencilled on the other sections at the same distances from the hydrochloric acid line. Then

10–15 consecutive parallel lines are pencilled at 0.5 cm. intervals on both sides of the provisional border line in each of these sections, followed by scissoring along those lines. The small-slivers thus given are separately cut into pieces, put into stoppered tubes in Fig. 1, and after addition of 2 cc. of water, subjected to the successive treatments with acetylacetone- and Ehrlich-reagent described above. It is to be remarked that 0.7 *N* Na_2CO_3 is used also here as the solvent for the acetylacetone reagent. After color development, the reaction mixtures are quickly freed from the paper fibers in a centrifuge and the extinctions at 537 mμ wave-length are measured. The real border between the two spots in each of the chromatograms lies in the small-sliver which gave the minimum coloration. A half of this extinction is added to the total sum of the extinctions given by the upper small-slivers and to that of the extinctions by the lower small-slivers to calculate the amount ratio of glucosamine to glactosamine. Glucosamine and galactosamine are recovered to the same extent by this chromatographic procedure if assumed that the extinctions for the two hexosamines are equal also in this case.

Fractionated Determination of the Hexosamines in Mixtures of Goldschmiedt-positive Mucopolysaccharides or of a Molisch-positive Mucopolysaccharide or Glucidamin and a Goldschmiedt-positive Mucopolysaccharide

The hydrolysis is effected with 6 *N* HCl at 100°C for 15 hours. The analysis is processed in the same way as above.

Explanatory Experiments

I. Concentration of HCl and Duration of Time for Hydrolysis of Mucopolysaccharides in Estimation of Total Hexosamines

A) 2.5 mg. portions of a group mucopolysaccharide preparation which Masamune *et al.*[3] had obtained from pig stomach mucus (N 4.1%, galactose 26.8%, L-fucose 12.0%, sialic acid 5.0%, ash 1.1%) were heated with 5 cc. of 2 *N* HCl (Blix)[4] (*a*), 2 cc. of 4 *N* HCl (Johnston *et al.*[5]) (*b*), 2 cc. of 6 *N* HCl (Gardell[6]) (*c*), 2.5 cc. of 0.5 *N* HCl (Rondle and Morgan[2]) (*d*) or 4 cc. of 1 *N* HCl (*e*) at 100°C for various durations of time, and the total hexosamine amount was determined according to the description above, after adjusting the NaCl concentration in the neutralized hydrolysate to 4.68%. The results are embodied in Table I.

B) 2.5 mg. portions of a previous specimen of mucoitin from naval cords[7] (N 3.2%, glucuronic acid 45.8%, ash 0.34%) were subjected to hydrolysis with agent *a*, *b* or *c* above, followed by the treatments in the

TABLE I

Hexosamine Analysis of Group Mucopolysaccharide from Pig Stomach Mucus, Umbilical Mucoitin and Sodium Chondroitinsulfate from Whale Nasal Cartilage, after Hydrolysis with Various Volumes of HCl of Various Concentrations for Various Durations of Time

a: 5 cc. of 2 *N* HCl; *b*: 2 cc. of 4 *N* HCl; *c*: 2 cc. of 6 *N* HCl; *d*: 2.5 cc. of 0.5 *N* HCl; *e*: 4 cc. of 1 *N* HCl. Figures express percentages of the materials.

Mucopolysaccharide	Time of hydrolysis (hrs.)	Hydrolysis agent				
		a	*b*	*c*	*d*	*e*
Group mucopolysaccharide	2	–	34.9	35.3	–	–
	4	–	35.6	34.8	30.3	–
	6	35.4	35.5	–	–	33.1
	8	–	35.0	–	31.0	–
	10	35.6	–	–	–	33.4
	15	35.4	–	–	31.1	–
	24	–	–	–	31.3	33.6
Mucoitin	2	–	36.7	39.9		
	4	29.0	40.2	39.7		
	8	34.2	39.6	39.4		
	15	37.1	39.2	38.5		
	24	36.9	–	–		
Sodium chondroitinsulfate	2	18.2	27.8	32.0		
	4	–	32.1	31.8		
	8	23.7	31.8	31.2		
	15	29.8	31.0	30.3		
	24	29.6	–	–		

second and succeeding stages above. The results are shown in Table I.

C) 2.5 mg. portions of sodium chondroitinsulfate[8] prepared according to Jorpes (N 7.6%, glucuronic acid 37.9%, ash 21.8%) were treated as in B). The values of the hexosamine given are embodied also in Table I.

The figures in the table indicate that hydrolysis with 2 cc. of 4 *N* HCl (*b*) for 4 hours give the maximum value irrespective of kind of mucopolysaccharide.

II. Relation between Concentration of Na_2CO_3 as Solvent of Actylacetone and Intensity of Color Developed

(A) 0.08 mg. portions as free bases of the hexosamine hydrochlorides were dissolved in 2 cc. of water or the same volume of 4.68% NaCl, and after treatment with 1 cc. of acetylacetone reagents prepared with Na_2CO_3 solutions of varying concentrations, subjected to further operations in the determination method of total hexosamines above. As shown in Table II, 0.5 and 0.7 normalities are the respective optimum

concentrations of Na_2CO_3 in the acetylacetone reagents to analyze the hexosamine solutions in water and those in 4.68% NaCl.

TABLE II

Relation between Concentration of Na_2CO_3 Solution as Solvent of Acetylacetone and Extinction ($-\log T$) of Color Developed with Ehrlich Reagent in Case of IIA of the Explanatory Experiments

Figures express extinctions at the wave-length of 537 mμ.

Concentration of Na_2CO_3 (normality)	Hexosamine dissolved in water		Hexosamine dissolved in 4.68% NaCl	
	Glucosamine	Galactosamine	Glucosamine	Galactosamine
0.3	0.320	0.323	0.321	0.325
0.5	**0.431**	**0.432**	0.420	0.422
0.7	0.426	0.425	**0.432**	**0.433**
0.9	0.411	0.407	0.421	0.418
1.2	0.319	0.305	0.321	0.310

B) 0.08 mg. portions as free bases of the hexosamine hydrochlorides were dissolved in 2 cc. of water, and stood in contact with cut pieces of a 0.5×4.5 cm. sliver, which had been cut out of a 4.5×56 cm. paper sliver irrigated in advance with solvent butanol-pyridine-dilute HCl (5:3:2 by volume). The mixtures were then subjected to the procedure of fractionated hexosamine analysis, employing acetylacetone reagents prepared with Na_2CO_3 solutions of various concentrations. The results are shown in Table III. Here 0.7 normality was found to be the concentration of the Na_2CO_3 solution giving the highest value of hexosamine.

TABLE III

Relation between Concentration of Na_2CO_3 Solution as Solvent of Acetylacetone and Extinction ($-\log T$) of Color Developed with Ehrlich Reagent in Case of IIB of the Explanatory Experiments

The solvents of the hexosamine solutions were water.

Concentration of Na_2CO_3 (normality)	Extinction at the wave-length of 537 mμ	
	Glucosamine	Galactosamine
0.3	0.196	0.205
0.5	0.330	0.330
0.7	**0.429**	**0.429**
0.9	0.418	0.414
1.2	0.383	0.377

III. Recovery of Glucosamine and Galactosamine from Their Mixtures by the Fractionated Analysis

In Table IV are shown the recoveries of the hexosamines in four

TABLE IV

Average Recoveries of Glucosamine and Galactosamine from Their Mixtures

No. of example	Amount sampled of the hydrochlorides as free bases (μg)		Recovery					
	Glucosamine	Galactosamine	Total hexosamines (μg)	$\frac{\text{Glucosamine}}{\text{Galactosamine}}$ ratio	Glucosamine μg	Glucosamine %	Galactosamine μg	Galactosamine %
1	70	70	140	1.00	70	100	70	100
2	45	225	272	0.19	44	98	229	102
3	300	30	333	10.10	303	101	30	100
4	170	85	254	2.02	170	100	84	99

examples. The average hexosamine amounts recovered from individual small-slivers are expressed in extinctions in Fig. 3.

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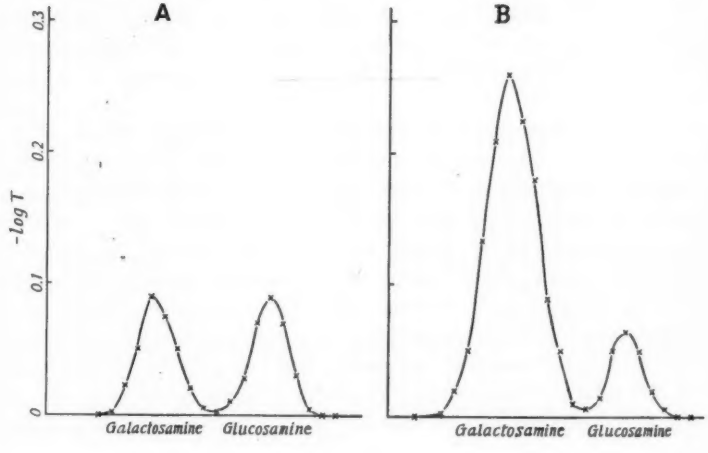
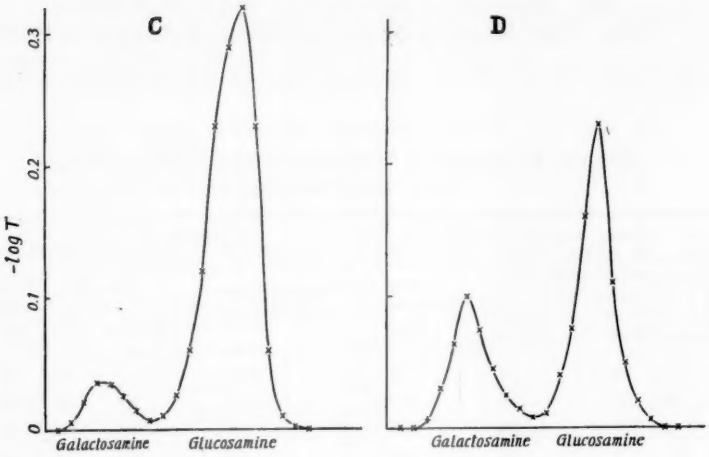
Fig. 3. Showing average recoveries of the hexosamines from the individual small-slivers in the examples in Table IV. The amounts of the hexosamines are expressed in extinctions. **A**: Ex. 1. **B**: Ex. 2. **C**: Ex. 3. **D**: Ex. 4.





References

1) Masamune & Yosizawa, Tohoku J. Exp. Med., 1956, **64**, 53.
2) Rondle & Morgan, Bioch. J., 1955, **61**, 586.
3) Masamune, Yosizawa & Haga, Tohoku J. Exp. Med., 1956, **64**, 257.
4) Blix, Acta chem. Scand., 1948, **2**, 467.
5) Johnston, Ogston & Stanier, Analyst, 1951, **76**, 88.
6) Gardell, Acta chem. Scand., 1953, **7**, 207.
7) Masamune & Yosizawa, Tohoku J. Exp. Med., 1952, **56**, 35.
8) Masamune, Yosizawa & Maki, *ibid.*, 1951, **55**, 47.

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Biochemical Studies on Carbohydrates

CXCV. Digestion of the Group O and AO Mucopolysaccharides from Pig Stomach Mucus by the O Enzyme from *B. fulminans*

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In 1953, Crumpton and Morgan[1] reported that the enzyme from *Cl. Welchii* cleaves off parts of all the sugar components of human and animal A, B, H and Le^a substances. In the next year, Watkins and Morgan[2] informed that the action of the H specific enzyme from *T. foetus* is inhibited by L-fucose and galactosamine. More recently, however, these authors found[3] that the action of the crude *T. foetus* extract on Group H substance (from ovarian cyst fluid) is inhibited by L-fucose only and that it contains an enzyme destroying galactosamine. The findings above in their laboratory led them to deduce that L-fucose which is a component of group substances plays the important role of H-activity.

On the other hand, Iseki's school[4]-[7] isolated an enzyme from *B. fulminans* which causes destruction of the Group O activity of O substances from human and pig stomachs with simultaneous fission of L-fucose. Furthermore Ikeda[8] one of Iseki's co-workers said that the O group mucopolysaccharide from human stomach loses the group activity with reduction of levorotatory power when digested with the extract of *T. foetus*.

The writer separated the O enzyme of *B. fulminans* according to Iseki and Masaki[5] to reexamine the action of the enzyme. The Group O and AO mucopolysaccharides from pig stomach mucus were applied as the substrates. The results obtained may be summarized as follows: 1) The action of the enzyme upon the Group O mucopolysaccharide was inhibited by L-fucose strongly. The other simple sugars examined were not or scarcely effective. 2) The enzyme disintegrated galactosamine but not acetylglucosamine nor acetylgalactosamine nor L-fucose. 3) More than half of L-fucose and a trace of galactose but no sugar components else became dialyzable when the group mucopolysaccharides were digested with the enzyme. The glucosamine/galactosamine ratios of the

group mucopolysaccharides lowered, whereas the hexosamines remained undecreased in total amount. This unexplainable fact will be left for a later study. The undialyzable residues possessed no optical rotatory power and reduced more iodine than the respective group mucopolysaccharides.

Experimental

Preparation of the O and AO Group Mucopolysaccharides from Pig Stomach Mucus

The O mucus (Group O active against anti-OS saliva chicken serum at a dilution of $1:>1\times10^5$ and Group A active against normal α serum at a dilution of $1:<5\times10^2$) and the AO mucus (Group O and A active against the antisera above at a dilution of $1:>1\times10^5$) were pooled and subjected to one[9] of the writer's procedures for preparation of the group mucopolysaccharide (The mucus is treated with 1 *N* NaOH at 0–3°C), and the substances given were further fractioned with $(NH_4)_2SO_4$ as follows. 3 g. of substance were dissolved in 20 cc. of water and saturated with $(NH_4)_2SO_4$ and the centrifugate was washed with 10 cc. of the sat. $(NH_4)_2SO_4$. The supernatant and washing were dialyzed together in a cellophane bag against running water for 3 days, distilled in vacuo to about 20 cc. and again treated with $(NH_4)_2SO_4$ similar to above in order to eliminate the remaining part of the $(NH_4)_2SO_4$-precipitable. The supernatant and washing here were dialyzed as above and after subsequent 3 days' electrodialysis against changes of water, condensed to 20 cc., followed by pouring into 10 volumes of 99% alcohol under agitation. The deposit was washed with 20 cc. of 99% alcohol and dried in vacuo over H_2SO_4. 2.2 and 2.8 g. of Group O and AO substances (white powders) were yielded respectively. They will be referred to as MP O and MP AO. These preparations were biuret negative and Molisch and direct- and indirect-Osaki-Turumi positive.

Properties of MP O and AO

Electrophoresis. The substances showed a single boundary after electrophoresis at pH 7.8 as well as after electrophoresis at pH 4.5 (Fig. 1).

Group activity. The glass plate method described by Masamune *et al.*[10] was applied for the measurement. The results are illustrated in Table I.

Quantitative analysis. The analytical figures are embodied in Table II.

Optical rotation in water:

$$\text{MP O:}\quad [\alpha]_D^{19}=\frac{-0.41\times100}{1.0\times1}=-41.0°$$

$$\text{MP AO:}\quad [\alpha]_D^{19}=\frac{-0.30\times100}{1.0\times1}=-30.0°$$

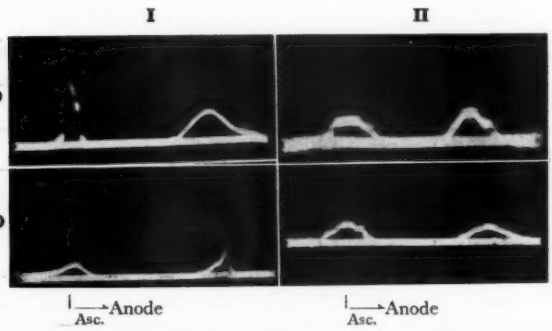

Fig. 1. Showing the electrophoretic patterns (4/5×) of 0.5% solutions of MP O (**O**) and AO (**AO**) in phosphate buffer pH 7.8, I 0.145 (**I**) and acetate buffer pH 4.5, I 0.2 (**II**) ; current 8 mA ; temp. 10°C. Exposure 60 minutes after starting current.

Preparation of the Enzyme Solution

The procedure of Iseki and Masaki[5] was adapted. The strain of *B. fulminans* kindly given by Prof. Iseki was cultivated, and 60 g. of the wet culture were shaken with 160 cc. of *M*/15 phosphate buffer of pH 7.0 at 37°C for 24 hours and centrifuged. The centrifugate was washed with 40 cc. of the buffer, and the turbid supernatant was filtered through Toyo Roshi filter paper No. 85 with suction. The filtrate and the washing of the centrifugate were united, chilled in ice and added to with 3 volumes of ice-chilled acetone. The deposit here was quickly separated in a centrifuge, freed from acetone by aeration and immediately taken up in 40 cc. of the phosphate buffer. To the centrifuged supernatant 18 g. of crystalline $(NH_4)_2SO_4$ were added to 6/10 saturation. The precipitate hereby was spun down and washed with 10 cc. of the 6/10 sat. $(NH_4)_2SO_4$ twice. It was reprecipitated from the phosphate buffer (60 cc.) by 6/10 saturation with $(NH_4)_2SO_4$, and after dissolving in 60 cc. of water, dialyzed in a cellophane bag against 500 cc. of water for 10–12 hours (The enzyme passes slowly through the membrane). The solution was precipitated with 3 volumes of acetone, freed from acetone and dissolved in 40 cc. of the phosphate buffer as above. And the centrifuged supernatant, which was transparent and light brown, was used as the enzyme solution in the following experiments.

Inhibitory Effect of Simple Sugars upon the O Enzyme

0.5 cc. portions of a 0.2% MP O solution in physiological saline (0.9%) were mixed with an equal volume of a 2% solution in the saline of L-fucose, acetylglucosamine, acetylgalactosamine, glucosamine hydro-

TABLE

Anti-hemagglutinative Group

Anti-OS : an anti-OS saliva chicken serum. G : a normal anti-O goat serum. marked hemaggl., ## : strong hemaggl.

Group mucopoly-saccharide	Red cells	Serum	Dilution of the							
			10^3	2×10^3	4×10^3	8×10^3	10^4	2×10^4	4×10^4	8×10^4
MP O	A	α	++	##	##	##	##			
	O	Anti-OS	–	–	–	–	–	–	–	–
	O	G	–	–	–	–	–	–	–	–
MP AO	A	α	–	–	–	–	–	–	–	–
	O	Anti-OS	–	–	–	–	–	–	–	–
	O	G	–	–	–	–	–	–	–	±

TABLE II

Analyses of MP O and AO

Analysis	In per cent		In molecular ratio	
	MP O	MP AO	MP O	MP AO
N*	4.2	4.2		
Total hexosamine†	36.4	35.6	2.0	2.0
Glucosamine†	29.9	25.7	1.7	1.4
Galactosamine†	6.5	9.9	0.4	0.6
Glucosamine/galactosamine ratio	4.6	2.6		
Galactose‡	27.5	25.1	1.5	1.4
L-Fucose§	13.6	13.0	0.8	0.8
Sialic acid//	1.4	2.6	0.04	0.08
Iodine use as glucose¶	4.5	3.6		
Ash**	1.2	1.6		

* Micro Kjeldahl. † Masamune and Yosizawa[11]. ‡ Masamune and Sakamoto[12]. § Masamune and Sakamoto[13]. // Werner and Odin[14] (Direct Ehrlich reaction); Calcd. as $C_{11}H_{19}NO_8$. ¶ Macleod and Robison[15]. ** Pregl method without use of H_2SO_4.

chloride, galactosamine hydrochloride, galactose, lactose, melibiose or acetyllactosamine. The mixtures were added to with 1 cc. of the enzyme solution, and after adjusting to pH 6.5 with ca. 5% acetic acid, incubated with two drops of toluene at 37°C for 2 days. The digestates were heated in a boiling water-bath for 5 minutes and centrifuged, and the supernatant were examined as to the group activities by the method applied above. The blank was carried out by replacing the sugar solution with the physio-

I

Activities of MP O and AO

− : no hemagglutination ; ± : faint hemaggl., + : slight hemaggl., ++ :

sustances (1:)									Dilution of the sera diluted to titre 16 in the control runs (1:)				
10^5	2×10^5	4×10^5	8×10^5	10^6	2×10^6	4×10^6	8×10^6	10^7	2	4	8	16	32
									++	++	++	±	−
−	−	−	−	−	−	+	+	++	++	++	+	±	−
+	+	+	+	+	++	++	++	++	++	++	++	+	−
−	−	−	−	−	−	−	+	+					
−	−	−	−	−	+	+	+	++					
+	+	+	+	+	++	++	++	++					

logical saline and the control by replacing the enzyme solution with the phosphate buffer. Table III shows the results. L-Fucose depressed the enzyme action with cartainty. The effect of galactosamine was scarcely recognized.

Action of the O Enzyme on Simple Sugars

0.5 cc. each of 2% solutions in the physiological saline of acetylglucosamine, acetylgalactosamine, glucosamine hydrochloride, galactosamine hydrochloride and L-fucose were mixed with 2 cc. of the enzyme solution, and after addition of 5% acetic acid to pH 6.5, incubated with 2 drops of toluene at 37°C for 2 days. Hexosamines were analyzed by Masamune and Yosizawa's improved method[11] and L-fucose according to Masamune and Sakamoto.[13] The results are embodied in Table IV.

Action of the O Enzyme upon MP O and AO

150 mg. of MP O and of MP AO were dissolved in 20 cc. of the phosphate buffer above, mixed with 30 cc. of the enzyme solution, and after addition of 0.5 cc. of about 5% acetic acid to pH 6.5 and 0.5 cc. of toluene to prevent putrefaction, maintained at 37°C for 3 days. The blank without the substrate was carried out in parallel. A 1 cc. portion of the mixtures were pipetted out, heated for 5 minutes and centrifuged. The supernatant were examined regarding Group A and Group O activities by the method above (Table V). No inhibition to hemagglutination occurred in the blank. Another 1 cc. portion of the digestates were diluted five times with water and the optical rotations were measured. The digestates of MP O and AO showed α values of −0.02 and −0.01° re-

TABLE

Inhibition of Simple Sugars to the Action of the O Enzyme Enzyme in the Presence

An anti-OS saliva chicken serum was used as the anti-serum. The symbols signify

Sugar added	Dilution of MP O							
	$2+10^3$	4×10^3	8×10^3	10^4	2×10^4	4×10^4	8×10^4	10^5
The blank without any sugar	−	−	−	−	−	−	−	−
The control without the enzyme	++	++	++	++	++	++	++	++
L-Fucose	−	−	−	−	−	−	−	−
Acetylglucosamine	++	++	++	++	++	++	++	++
Acetylgalactosamine	++	++	++	++	++	++	++	++
Glucosamine hydrochloride	++	++	++	++	++	++	++	++
Galactosamine hydrochloride	+	+	+	±	±	+	+	+
Galactose	++	++	++	++	++	++	++	++
Lactose	++	++	++	++	++	++	++	++
Melibiose	++	++	++	++	++	++	++	++
Acetyllactosamine	++	++	++	+	+	++	++	++

TABLE IV

Action of the O Enzyme on Simple Sugars

Sugar	Amount incubated with the enzyme (mg.)	Amount remaining undecomposed (mg.)	Recovery (%)
Acetylglucosamine	8.1	8.1	100
Acetylgalactosamine	8.1	8.0	99
Glucosamine hydrochloride	8.3	8.2	99
Galactosamine hydrochloride	8.3	7.6	91
L-Fucose	10.0	9.9	99

TABLE

Action of the O Enzyme on MP O and AO (Group Activities of the

Anti-OS and −~+++ signify the same as in Table I.

Group mucopolysaccharide	Red cells	Serum	Dilution of the digestates							
			10^3	2×10^3	4×10^3	8×10^3	10^4	2×10^4	4×10^4	8×10^4
MP O	A	α	+++	+++	+++	+++				
	O	Anti-OS	++	++	++	+	++	+++	+++	+++
MP AO	A	α	−	−	−	−	−	−	−	−
	O	Anti-OS	++	++	++	++	++	+++	+++	+++

III

(The Group O Activities of MP O after Digestion with the
of Varied Simple Sugars)

the same as in Table I.

digested (1:)							Dilution of the serum diluted to titre 16 in the control run (1:)				
2×10^5	4×10^5	8×10^5	10^6	2×10^6	4×10^6	8×10^6	2	4	8	16	32
−	−	−	−	±	+	+	##	##	##	±	−
##	##	##	##	##	##	##					
−	−	+	+	+	+	##					
##	##	##	##	##	##	##					
##	##	##	##	##	##	##					
##	##	##	##	##	##	##					
+	+	##	##	##	##	##					
##	##	##	##	##	##	##					
##	##	##	##	##	##	##					
##	##	##	##	##	##	##					
##	##	##	##	##	##	##					

spectively in a 1 dm. tube which lie within the limits of measurement error. The enzyme solution showed no optical rotation. The balances of the digestates were dialyzed in a cellophane bag for 3 days against ten 300 cc. changes of water, and the inner fluids and dialysates were each distilled to less than 25 cc. and made up accurately to 25 cc. to estimate the sugars in them. The corresponding condensates in the blank were also analyzed. Results obtained are expressed in per cent of the substrates in Table VI.

V

Group Mucopolysaccharides after Digestion with the Enzyme)

as converted into the substrates (1:)									Dilution of the sera diluted to titre 16 in the control runs (1:)				
10^5	2×10^5	4×10^5	8×10^5	10^6	2×10^6	4×10^6	8×60^6	10^7	2	4	8	16	32
									##	##	+	+	−
##	##	##	##	##	##	##	##	##	##	##	+	±	−
−	−	−	−	−	−	±	+	+					
##	##	##	##	##	##	##	##	##					

TABLE VI

Analyses of the Digestion Products of MP O and of MP AO

The figures express percentages of the substrates MP O and AO, excepting those in parentheses which show molecular ratios.

Analysis	Digestion products of MP O		Digestion products of MP AO	
	Undialyzable	Dialyzable	Undialyzable	Dialyzable
Total hexosamine†	35.8 (2.0)	0.0	35.1 (2.0)	0.0
Glucosamine†	23.0 (1.3)		23.0 (1.3)	
Galactosamine†	12.8 (0.7)		12.1 (0.7)	
Glucosamine/galactosamine	1.8		1.9	
Galactose‡	26.6 (1.5)	1.3 (0.07)	24.6 (1.4)	1.0 (0.06)
L-Fucose§	4.6 (0.3)	8.6 (0.5)	5.6 (0.3)	7.0 (0.4)
Sialic acid//	1.2 (0.04)	<0.2	2.3 (0.07)	<0.2
Iodine use as glucose¶	6.2		5.1	

†, ‡, §, //, ¶ Signify the same as in Table II.

SUMMARY

1. The action of the O enzyme from *B. fulminans* (Iseki and Masaki) upon the Group O and AO substances from pig stomach mucus was inhibited undoubtedly by L-fucose.

2. The enzyme decomposed galactosamine slightly.

3. The enzyme destroyed Group O activity of the Group O and AO mucopolysaccharides, with cleaving off of about 2/3 and 1/2 respectively of L-fucose, deprivation of rotatory power and decrease of glucosamine/galactosamine ratio. The undialyzable digestion products used more iodine than the respective substrates.

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References

1) Crumpton & Morgan, Bioch. J., 1953, **54**, xxxii.
2) Watkins & Morgan, Brit. J. Exp. Pathol., 1954, **35**, 181.
3) Watkins & Morgan, *ibid.*, Nature, 1955, **175**, 676.
4) Iseki & Tsunoda, Igaku to Seibutsugaku (Jap.), 1952, **24**, 96.
5) Iseki & Masaki, *ibid.*, 1954, **30**, 66.
6) Masaki, *ibid.*, 1955, **34**, 269.
7) Masaki, *ibid.*, 1955, **36**, 109.
8) Ikeda, *ibid.*, 1955, **37**, 8.
9) Yosizawa, Tohoku J. Exp. Med., 1954, **61**, 45.
10) Masamune, Hakomori, Numabe, Akama & Kamiyama, *ibid.*, 1955, **61**, 283.
11) Masamune & Yosizawa, *ibid.*, this volume, p. 169.

12) Masamune & Sakamoto, *ibid.*, 1956, **63**, 345.
13) Masamune & Sakamoto, *ibid.*, 1956, **63**, 363.
14) Werner & Odin, Acta soc. med. Upsal., 1952, **57**, 230.
15) Macleod & Robison, Bioch. J., 1929, **23**, 517.

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Biochemical Studies on Carbohydrates

CXCVI. Comparison among Blood Group A, B and O Mucopolysaccharides from Human Gastric Juices

By

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In 1949, Tiba and others[1] separated group mucopolysaccharides from human gastric juices, and the substances given were of resembling compositions but the A substance differed in optical rotation from the B and O substances, which were similar in this respect on their side. The writers have isolated by a milder procedure the group substances in an electrophoretically homogeneous state to reexamine their chemical nature employing recent methods and also to see the action of the Group O enzyme from *B. fulminans* upon them. The results may be summarized: 1) The glucosamine/galactosamine ratios of the Group A, B and O substances are 1.8, 3.4 and 3.0 respectively. The mutual relations of these ratios are in accord with those found by Leskowitz and Kabat[2] in human A, B and O substances and pig A and O substances, those found by Rondle and Morgan[3] in A, B and O substances from ovarian cyst fluid and that found by Yosizawa[4] in A and O substances from pig stomach mucus. 2) No significant quantitative differences exist among the corresponding sugar components of the group mucopolysaccharides, hexosamines and sialic acid excepted. The sialic acid content of the B mucopolysaccharide exceeds the corresponding contents of the other group mucopolysaccharides. 3) Specific rotations of the A, B and O substances are $+8.3°$, $-12.0°$ and $-9.2°$. These values diverge from the corresponding values of Tiba and others' preparations despite of their similarity in reciprocal connections. 4) When digested with the O enzyme from *B. fulminans*, the O activity of not only the Group O substance but also the Group A and B substances was lost. At the same time, the glucosamine/galactosamine ratios decreased, notwithstanding that the hexosamines remained unreduced as expressed in total amount. In addition, a part of L-fucose and a trace of galactose were split off from the group substances. These findings

coincide with Yosizawa's[5] in the pig Group O and AO substances.

Experimental

Preparation of the Group Mucopolysaccharides

1*st stage*. Gastric juices were obtained from non-cancerous patients by the method of Katsch and Kalk and pooled according to the blood group A, B or O of the individuals. Each batch was made nearly clear by passing through a gauze at first and then through a filter paper. The filtrate was neutralized, dialyzed in a cellophane bag against running water for 3 days and distilled in vacuo to a thick solution, then the condensate was precipitated by pouring in small portions into 10 volumes of abs. alcohol under agitation. The deposit was washed with alcohol and dried in vacuo over anhydrous calcium chloride. Yielded were 4.8 g. of a powder from the gastric juices of 55 Group A persons, 3.4 g. of a powder from those of 42 Group B persons and 4.05 g. of a powder from those of 56 Group O persons. The powders were fractioned by a modification of Aminoff and others' procedure,[6] as will be exemplified with the Group A powder.

2*nd stage*. 4.75 g. of the powder were taken up in 100 cc. of water. The small insoluble part was centrifuged off and washed with 50 cc. of water. The solution and washing were combined together and made up to 160 cc. with water and 80 cc. of 10% trichloroacetic acid were added. After 30 minutes of standing, the precipitate was separated and washed with 10 cc. of 3.3% trichloroacetic acid—**Ppt. 1**. The mother fluid and washing here were added to with 1.5 volumes of acetone under agitation, stood for 1 hour and centrifuged. The centrifugate was washed 3 times with 40 cc. of a 3.3% trichloroacetic acid-acetone mixture in the same volume proportion as the supernatant and 3 times with 40 cc. of acetone and dried as above. 2.1 g. of a gray powder (**Ppt. 2**) were given.

The mother fluid of Ppt. 2 was added to with an equal volume of acetone and the deposit was centrifuged, washed with acetone and dried—**Ppt. 3**. The supernatant was distilled in vacuo to 240 cc. in order to expel off most of the acetone and then dialyzed to remove trichloroacetic acid, followed by vacuum distillation to 80 cc. Then it was precipitated with 3 volumes of acetone and washed with acetone—**Ppt. 4**. The mother fluid of this precipitate was added to with additional 560 cc. of acetone. The deposit here was separated in a centrifuge, washed with acetone and ether and dried (**Ppt. 5**). The supernatant was distilled under reduced pressure to 2–3 cc. and precipitated with 50 cc. of abs. ethanol containing anhydrous sodium acetate, and after washing with ethanol and ether, dried—**Ppt. 6**. Group A activity of Ppts. 1–6 were measured by the glass plate method described by Masamune *et al.*[7] with following results.

Ppt.	Group A active dilution (1 :)
1	2.56×10^4
2	1×10^8
3	4.096×10^5
4	4×10^2
5	1×10^2
6	1×10^2

3rd stage. 2.0 g. of Ppt. 2 were taken up in 50 cc. of the acetate buffer of pH 4.6 and I 0.4 and the insoluble part was rejected. The transparent, viscid solution was chilled in ice and 40 cc. of 99% alcohol were added to 44%. The solution which turned turbid was stood in a refrigerator (−5°C) overnight and centrifuged in a refrigerated centrifuge (−10°C). The centrifugate was again precipitated from the acetate buffer with alcohol and centrifuged off in a similar manner. The two successive supernatant fluids were united, and after addition of additional 20 cc. of 99% alcohol to 50%, placed in the refrigerator. The deposit, centrifuged off as above, was washed with 40 cc. of abs. alcohol twice and dried in vacuo over $CaCl_2$. 210 mg. of a white powder were given.

4th stage. 200 mg. of it were exhausted with two 40 cc. portions of 90% phenol. The insoluble residue was washed thoroughly with 20 cc. portions of 99% alcohol to eliminate the adhering phenol, and after washing with 40 cc. of ether, dried as above. The white powder given (the Group A mucopolysaccharide, **MP A**) amounted to 135 mg.

70 mg. of the Group B mucopolysaccharide (**MP B**) and 79 mg. of the Group O mucopolysaccharide (**MP O**) were obtained by fractioning 3.35 g. and 3.9 g. of the respective materials similar to above.

All of the preparations were Molisch and direct- and indirect-Osaki-Turumi positive and almost biuret negative.

Properties of the Preparations

Electrophoresis. They were examined in a Tiselius apparatus and proved homogeneous (Fig. 1).

Group activity. It was assayed by the glass plate method as above. As shown in Table I, MP A and B were Group O active, though less strong than their Group A or B potency.

Paper partition chromatography of the sugar components. 5 mg. of substance were hydrolyzed by heating with 0.5 cc. of 1 *N* H_2SO_4 at 100°C for 5 hours. The hydrolysate was neutralized with the sat. $Ba(OH)_2$ to pH 6.4, and the centrifuged supernatant and the washing of the centrifugate were distilled together in vacuo to dryness. The still-residue was dissolved in 0.04 cc. of water, and 0.02 cc. of the solution was paper-chromatographed according to Masamune and Yosizawa's description.[8]

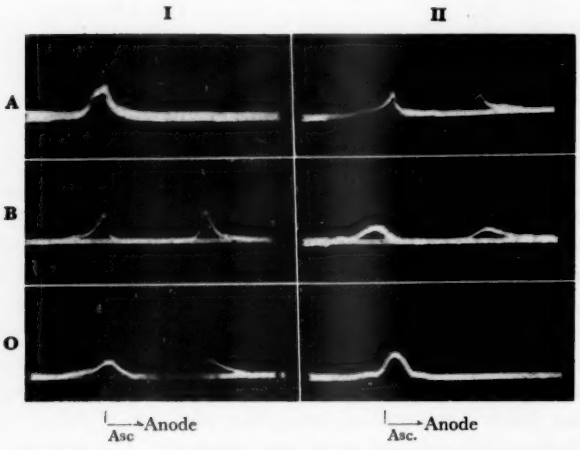

Fig. 1. Showing the electrophoretic patterns (4/5×) of 0.5% solutions of MP A (**A**), MP B (**B**) and MP O (**O**) in phosphate buffer pH 7.7, I 0.2 (**I**) and acetate buffer pH 4.6, I 0.2 (**II**). Current 8 mA. Temperature of test: **IA** 18°; **IB** 12°; **IO** 18°; **IIA** 10°; **IIB** 7°; **IIO** 9°. Time till exposure after starting current: **IA**, **IB** and **IO**, 50 minutes; **IIA**, **IIB** and **IIO** 60 minutes. Mobility expressed in $cm^2 \cdot volt^{-1} \cdot sec^{-1} \times 10^5$: **IA** 2.30; **IB** 2.05; **IO** 2.39; **IIA** 3.01; **IIB** 3.32; **IIO** 2.66.

TABLE

Anti-hemagglutinative Group Potencies

Anti-OS: an anti-OS saliva chicken serum. − : no hemagglutination, ± : faint

Mucopoly-saccharide	Red cells	Anti-serum	Dilution of the					
			10^3	10^4	10^5	10^6	10^7	10^8
MP A	A	α	−	−	−	−	−	−
	B	β	+	++	+++	+++		
	O	Anti-OS	−	−	−	+	+	+
MP B	A	α	±	++	+++	+++		
	B	β	−	−	−	−	±	+
	O	Anti-OS	−	−	−	+	+	++
MP O	A	α	±	++	+++	+++		
	B	β	++	++	+++	+++		
	O	Anti-OS	−	−	−	−	−	−

Galactosamine, glucosamine, galactose and L-fucose were detected in each of the group mucopolysaccharides (Fig. 2).

Quantitative analysis. The analytical figures are embodied in Table II.

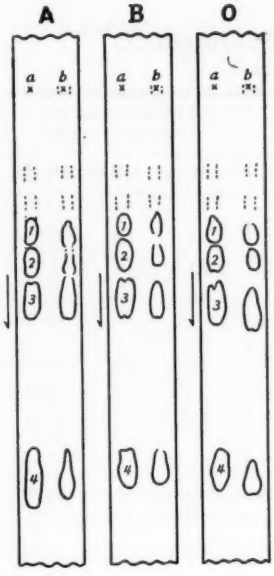

Fig. 2. Chromatograms (1/5×) of the hydrolysates of MP A (**A**), MP B (**B**) and MP O (**O**) developed by descending irrigation with solvent butanol-pyridine-water (5:3:2 by volume). Duration and temperature of test 24 hrs. and 25°C respectively. Indicator the aniline hydrogen phthalate reagent of Partridge. The standard sugar solution was 2% with respect to individual sugars and 0.01 cc. portions were sampled.

a : reference run ; b : main run. 1 : galactosamine ; 2 : glucosamine ; 3 : galactose ; 4 : L-fucose.

I

of the Group Mucopolysaccharides

hemaggl., + : slight hemaggl., ++ : marked hemaggl., +++ : strong hemaggl.

substances (1:)								Dilution of the diluted antisera of titre 16 in control runs (1:)				
10^{9}	10^{10}	10^{11}	10^{12}	10^{13}	10^{14}	10^{15}	10^{16}	2	4	8	16	32
−	−	−	−	−	−	−	±	+++	++	++	+	−
++	++	++	++	++	++	+++	+++	+++	++	+	±	−
								+++	++	+	±	−
++	++	++	++	++	+++	+++	+++					
++	+++	+++	+++									
−	−	+	+	++	++	++	+++					

Optical rotation in 0.005 N NaOH.

MP A: $[\alpha]_D^{19}=\frac{0.09\times 100}{1.08\times 1}=+8.3°$

MP B: $[\alpha]_D^{19}=\frac{-0.12\times 100}{1.00\times 1}=-12.0°$

TABLE II

Composition of the Group Mucopolysaccharides from Human Gastric Juices

Analysis	In per cent			In equivalents per epuivalent weight		
	MP A	MP B	MP O	MP A	MP B	MP O
N*	4.7	4.9	4.7			
Totol hexosamine†	30.8	29.0	30.0	1.7	1.6	1.7
Glucosamine†	19.8	22.4	22.5	1.1	1.3	1.3
Galactosamine†	11.0	6.6	7.5	0.6	0.4	0.4
Glucosamine/Galactosamine ratio	1.8	3.4	3.0			
Galactose‡	26.6	27.8	26.9	1.5	1.5	1.5
L-Fucose§	14.0	12.9	14.1	0.9	0.8	0.9
Sialic acid//	3.0	5.2	3.5	0.09	0.15	0.10
Ash¶	0.0	0.0	0.0			

* Micro Kjeldahl. † Masamune and Yosizawa.[9] ‡ Masamune and Sakamoto.[10] § Masamune and Sakamoto.[11] // Werner and Odin (Direct Ehrlich reaction)[12]; Calcd. as $C_{13}H_{21}NO_{10}$. ¶ Pregl method without use of H_2SO_4.

MP O: $[\alpha]_D^{19}=\frac{-0.09\times 100}{0.98\times 1}=-9.2°$

The solutions in water were not transparent enough for the measurement.

Action of the O Destroying Enzyme on the Group Mucopolysaccharides

The solution of the O enzyme from *B. fulminans* that had been prepared by Yosizawa[5] was used. 20 mg. each of the group mucopolysaccharides were dissolved in 5 cc. of the physiological saline (0.9% NaCl), added to with 5 cc. of the enzyme solution and adjusted to pH 6.5 with dilute acetic acid. The mixtures were incubated with a few drops of toluene for 3 days at 37°C. Then, a 0.6 cc. portion of the digestates were diluted with an equal volume of the physiological saline and placed in a boiling water-bath for 5 minutes, followed by cooling in water to examine the group activities using 0.2 cc. for each determination instead of 0.4 cc. As shown in Table III, all the mucopolysaccharides lost their Group O activity. The balance of the digestates were made up to 30 cc. with water and dialyzed for 3 days against ten 200 cc. changes of distilled water. The solutions and dialysates were each distilled in vacuo to 2–3 cc., made up accurately to 5 cc. with water and subjected to sugar analyses. The blank without the substrate was carried out in parallel, and the analytical

TABLE III

Anti-hemagglutinative Potencies of the Group Mucopolysaccharides after Digestion with the O Destroying Enzyme

Anti-OS and −∼## signify the same as in Table I.

Mucopolysaccharide subjected to digestion	Red cells	Anti-serum	Dilution of the digestates as converted into the substrates (1:)														Dilution of the diluted antisera of titre 16 in control runs (1:)				
			10^3	10^4	10^5	10^6	10^7	10^8	10^9	10^{10}	10^{11}	10^{12}	10^{13}	10^{14}	10^{15}	10^{16}	2	4	8	16	32
MP A	A	α	−	−	−	−	−	−	−	−	−	−	−	−	−	+	##	##	##	+	−
	B	β	+	##	##	##	##										##	##	+	±	−
	O	Anti-OS	##	##	##	##	##										##	##	+	±	−
MP B	A	α	+	##	##	##	##														
	B	β	−	−	−	−	+	+	##	##											
	O	Anti-OS	##	##	##	##	##														
MP O	A	α	+	##	##	##	##														
	B	β	##	##	##	##	##														
	O	Anti-OS	##	##	##	##	##														

figures obtained in this run were subtracted from the corresponding figures in the main runs. The results are shown in Table IV.

TABLE IV

Composition of Undialyzable Residue and the Amounts of the Dialyzable Split Products of the Group Mucopolysaccharides after Digestion with the O Destroying Enzyme

Figures express percentages of the group mucopolysaccharides subjected to digestion, excepting those in parentheses which show the equivalents per equivalent weight.

Analysis	MP A		MP B		MP O	
	Undialyzable residue	Dialyzable products	Undialyzable residue	Dialyzable products	Undialyzable residue	Dialyzable products
Total hexosamine†	30.0 (1.7)	0.0	28.4 (1.6)	0.0	29.0 (1.6)	0.0
Glubosamine†	17.5 (1.0)		20.5 (1.1)		20.2 (1.1)	
Galactosamine†	12.5 (0.7)		7.9 (0.4)		8.8 (0.5)	
Glucosamine/Galactosamine ratio	1.4		2.6		2.3	
Galactose‡	26.1 (1.5)	0.4 (0.02)	27.5 (1.5)	0.6 (0.03)	26.5 (1.5)	0.7 (0.04)
L-Fucose§	12.3 (0.8)	1.1 (0.07)	11.7 (0.7)	0.9 (0.05)	9.8 (0.6)	3.5 (0.2)
Sialic acid//	2.5 (0.07)	<0.2	4.7 (0.13)	<0.2	3.4 (0.10)	<0.2

†, ‡, §, // Singnify the same as in Table II.

SUMMARY

1. The Group A, B and O mucopolysaccharides differ from one another in glucosamine/galactosamine ratio. The Group A and O substances resemble each other and the Group B substance diverges from them in sialic acid content. The corresponding analytical figures of the other sugar components are similar to one another among the three mucopolysaccharides. As for optical rotation, the Group A substance is dextrorotatory, while the B and O substances are levorotatory and that to like extents.

2. The O enzyme from *B. fulminans* destroys the O activity of all the group mucopolysaccharides, accompanied by splitting off of a part of L-fucose and reduction of glucosamine/galactosamine ratio.

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References

1) Tiba, Teramura & Gotoh, Tohoku J. Exp. Med., 1949, **50**, 287; Tiba, *ibid.*, 1949, **50**, 293; 1949, **51**, 309.
2) Leskowitz & Kabat, J. Am. Chem. Soc., 1954, **76**, 4887.
3) Rondle & Morgan, Bioch. J., 1955, **59**, xiii.
4) Yosizawa, Tohoku J. Exp. Med., 1956, **64**, 253.
5) Yosizawa, *ibid.*, this volume, p. 177.
6) Aminoff, Morgan & Watkins, Bioch. J., 1950, **46**, 426.
7) Masamune, Hakomori, Numabe, Akama & Kamiyama, Tohoku J. Exp. Med., 1955, **61**, 283.
8) Masamune & Yosizawa, *ibid.*, 1953, **59**, 1.
9) Masamune & Yosizawa, *ibid.*, this volume, p. 169.
10) Masamune & Sakamoto, *ibid.*, 1956, **63**, 345.
11) Masamune & Sakamoto, *ibid.*, 1956, **63**, 363.
12) Werner & Odin, Acta soc. med. Upsal., 1952, **57**, 230.

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Biochemical Studies on Carbohydrates

CXCVII. Glucidamins in Yoshida Sarcoma

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Molisch-giving high-molecular components excepting the pentose-nucleic acid were sought in Yoshida sarcoma. Three glucidamins were isolated. Their yields, however, were very small probably because of the presence of large amounts of nucleic acids and a hexuronic acid-containing mucopolysaccharide or mucopolysaccharides from which they had to be liberated. No Molisch-giving mucopolysaccharide was detected.

Experimental

Fractionation was carried out, taking the result of Molisch test as the main indication.

First stage. The tumor knods on omentum majus of rats transplanted with Yoshida sarcoma were collected and preserved in 4 volumes of methanol for various durations of time. They were squeezed and dried at first in a Faust apparatus, and after pulverizing in an iron mortar, further dried in a vacuum-desiccator containing $CaCl_2$. The material given weighed 1,040 g.

Second stage. A 250 g. portion of it was shaken with 10 volumes of water at 7–14°C for 72 hours, employing a few drops of toluene to avoid putrefaction. The residue was further treated in a similar manner with 7.5, 5 and 5 volumes of water in succession. The fourth solution was only slightly Molisch positive. The somewhat opalescent solutions were added to with 30% acetic acid to pH 4.2 and centrifuged. The centrifugates which were Molisch negative or faintly Molisch positive were discarded. The slightly turbid supernatant fluids were each distilled in vacuo to about 1/30 the volume and kept in a refrigerator. They were then combined and dialyzed against running water for two days. The precipitate that occurred during the dialysis was rejected, and the clear fluid was condensed to a syrup, precipitated with an excess of ethanol, and after washing with ethanol (Sodium acetate was added to the ethanol when

necessary), dried in a desiccator. 6.1 g. of a white powder with brownish hue were given. After repetition of this procedure, 25.8 g. of substance were yielded from the whole material—**Fr. A.**

Third stage. 8.6 g. of it were taken for one run. It was dissolved in 100 volumes of water and acidified to pH 4.2 by means of 30% acetic acid as above to remove the part insoluble at this pH. The clear centrifuged supernatant was added to with 1/4 the volume of 20% trichloroacetic acid under agitation. After standing in a refrigerator for several hours, the voluminous flocculent precipitate was centrifuged off, and the transparent supernatant here was dialyzed against running water for 2 days until the pH of the solution was raised to 5.6 and distilled in vacuo to a syrup, which was precipitated with excessive ethanol and dried after washing with several changes of ethanol. Yield 2.1 g. The products after three runs, which weighed 6.2 g. in total, were united—**Fr. B.**

Attempts failed to free the trichloroacetic acid-precipitate from nucleic acids.

Fourth stage. Fr. B was Neuberg-Saneyoshi positive and its solution showed strong absorption at the wave-length of 260 mμ characteristic of nucleic acids. Therefore an orientating experiment was made with a part of the substance to see if liberation from polyuronic and nucleic acids is achievable, as follows.

0.6 g. of it was taken up in 100 volumes of water, and crystalline barium acetate was added to 3%. The solution that became cloudy was placed in a refrigerator for several hours, and the centrifuged supernatant was added to with ice-chilled ethanol to 15% and again stood in the refrigerator for several hours. The precipitate was quickly separated by centrifugation, and the clear supernatant was further subjected to fractionations in a similar manner, adding additional volumes of ethanol. The precipitates were separately dissolved in 100 volumes of water and added to with the sat. Na_2SO_4 just sufficient to carry down all the barium, and the filtrates were each dialyzed until no more SO_4-ions were detected, condensed and precipitated with alcohol.

Division	Conc. (%) of ethanol where the Division was pptd.	Yield (mg.)
1*	0	33
2	0–15	83
3	15–30	53
4	30–45	25
5	45–60	42
6†		250

* The precipitate that occurred on addition of barium acetate. † The fluid containing 60% ethanol was dialyzed, condensed to a small volume and precipitated with a large volume of ethanol.

All of the divisions showed the positive Molisch reaction and absorption at the wave-length of 260 mμ. The absorption was strongest in Div. 1 and became weaker and weaker with increase of the Division number, but even Div. 6 which was not precipitated by alcohol at 60%

concentration showed the absorption though weak. Hence fractionation was continued in a similar manner on 220 mg. of that last division. The subdivisions were given with yields:

Subdivision	Conc. (%) of ethanol where the Subdiv. was pptd.	Yield (mg.)
1′	0–45	40
2′	45–60	5
3′*		93

* The part soluble in 60% ethanol was recovered.

It was also found that the higher the ethanol concentration was, the subdivision precipitated showed the less strong absorption. Subdiv. 3′ was almost free from nucleic acids and gave Goldschmiedt reaction scarcely.

Therefore, the balance of Fr. B was treated in the same way and 0.72 g. of a fraction corresponding to Subdiv. 3′ was obtained—**Fr. C.**

Balances of Divs. 1–5 and Subdivs. 1′–2′ were combined and subjected to Kerr and Seraidarian[1] procedure and treatments with cupric chloride and lead acetate. But the mixture could not be perfectly freed from nucleic acids.

Fifth stage. Since biuret reaction of Fr. C was very strong in contrast to Molisch reaction, an orientating experiment was again processed in advance:

130 mg. of Fr. C were taken up in 10.3 cc. of 90% phenol and the centrifuged supernatant (transparent brownish) was fractioned with increasing volumes of ethanol at room temperature. The precipitates were separately washed with abs. ethanol and ether thoroughly, until they smelled no more of phenol. The substances given were white powders. The yields of them will be tabulated together with the results of their Molisch and biuret tests.

Division	Conc. (%) of ethanol where the Div. was pptd.	Yield	Molisch	Biuret
i*	0	0		
ii	0–10	0		
iii	10–20	0		
iv	20–30	33	++	−
v	30–40	24	+	+
vi	40–50	25	±	++
vii	50–60	18	−	++
viii†		29	−	++

* The insoluble part in 90% phenol. † The supernatant of Div. vii was precipitated with an excess of ethanol containing some sodium acetate.

Divs. iv and v which were Molisch positive were combined and again taken up in 5.5 cc. of 90% phenol and the solution was further fractioned in a similar manner. The results here will be also tabulated:

Subdiv.	Conc. (%) of ethanol where the Subdiv. was pptd.	Yield	Molisch	Biuret
i'*	0	23	+	−
ii'	0–10	0.6	++	−
iii'	10–20	1.2	+++	±
iv'	20–30	4.2	+++	±
v'	30–40	8.3	+	++
vi'	40–50	3.3	±	++
vii'	50–60	6.5	−	+++
viii'†		7.4	−	+++

* The 90% phenol-insoluble (This subdivision will be referred to as **Fr. D_1**). † The supernatant of Subdiv. vii' was precipitated with an excess of ethanol containing sodium acetate.

Based on the finding, the balance of Fr. C (590 mg.) was treated as above. 382 mg. of a white powder were obtained after precipitation at ethanol concentration range of 0–50% in the primary fractionation, and 85 mg. of this fraction remained undissolved in 90% phenol (**Fr. D_2**) and 253 mg. were precipitated from the solution by addition of ethanol to 50% in the secondary fractionation. The latter fraction in the secondary fractionation was again taken up in 90% phenol and after setting aside the 90% phenol-insoluble part (23.5 mg., **Fr. D_3**), the portion which precipitated on addition of ethanol to the solution up to 30% (68 mg.; **Fr. E**) and that which precipitated on increasing the ethanol concentration to 45% (112.1 mg.; **Fr. F**) were separated.

Sixth stage. a) Frs. D_1–D_3, which were insoluble in 90% phenol, (131.5 mg. in total) were united, washed exhaustively with 90% phenol, alcohol and ether and dried. Then, the mixture was dissolved in 13 cc. of water and alkalinized with NaOH to pH 9.0, whereby a precipitate occurred. The centrifugate, which remained mostly as an ash on ignition, was discarded, and the clear supernatant was neutralized, condensed and precipitated with ethanol. 74 mg. was the substance given. A part of it was dissolved in 100 volumes of acetate buffer of pH 4.6 and I 0.2 and examined electrophoretically by sending 10 mA current at 14°C. The solution showed a main slowly-moving boundary with mobility of 3.24×10^{-5} cm²/volt/sec and an accessory boundary quickly moving with mobility of 29.0×10^{-5} cm²/volt/sec, so that Masamune and others' electrophoretic sedimentation method[2] of fractionation was applied to the balance of the substance. Because of shortage of material, the fractionation was limited to a single run. The slowly-moving fraction separated (**Prep. I**) was 43.1 mg. The quickly-moving amounted to 14.9 mg. and proved Molisch faintly positive, direct-Osaki-Turumi negative, indirect-Osaki-Turumi faintly positive, Neuberg-Saneyoshi markedly positive and biuret negative.

b) Purification of Fr. F (Purification of Fr. E was postponed because of its small amount). Fr. F still showed several boundaries when its 1% solution in acetate buffer of pH 4.6 and I 0.2 was examined in a Tiselius apparatus. Hence, it was again fractioned by the procedure in the fourth stage. Namely, 98 mg. of it were dissolved in 10 cc. of water containing 0.3 g. of crystalline barium acetate, and ethanol was added to 60%. The precipitate was discarded, because it looked to be chiefly nucleic acids. The mother fluid was distilled in vacuo to dryness. The still-residue was dissolved in 100 cc. of water and passed through a 1.2×7 cm. column of Amberlite IR-120, then the effluent was condensed to a syrup, precipitated with ethanol and dried—**Prep. III.** Yield 61.3 mg. It was devoid of phosphorus and showed a single boundary after electrophoresis as will be detailed below.

c) Purification of Fr. E. It was at once fractioned with alcohol in the presence of barium acetate as above. And 34.5 mg. of the substance unprecipitable by ethanol at 60% were recovered also as above—**Prep. II.**

Properties of the Preparations

Electrophoresis. Acetate buffer of pH 4.6 and I 0.2 and 0.02 *M* veronal buffer of pH 8.5 whose ionic strength was adjusted to 0.2 with NaCl were used as the solvents. As shown in Fig. 1, all of Preps. I, II and III proved essentially homogeneous in this respect.

Solubility and precipitability. Prep. I. It dissolved in water, giving a slightly turbid solution which became clear on acidification to pH 4.5 and became slightly more turbid on alkalinization to pH 9.0, indicating that the inorganic contamination had not been perfectly removed at the final stage of preparation. The solution in plain water was neither coagulated by heating nor precipitated with barium acetate, cupric chloride and sulfosalicylic acid and by half saturation with $(NH_4)_2SO_4$, but was mostly precipitated by full saturation with $(NH_4)_2SO_4$. The substance did not dissolve in 90% phenol and glacial acetic acid at all.

Prep. II. It was readily soluble in water, but insoluble in glacial acetic acid. It was not precipitated from water with barium acetate and with cupric chloride.

Prep. III. It dissolved easily in water and was not precipitated by heating, by addition of barium acetate and cupric chloride, by saturation with $(NH_4)_2SO_4$ and by varying the pH of the solution within the range from 1 to 9, but was precipitated partly by dropping in of 20% sulfosalicylic acid and mostly by saturation with $(NH_4)_2SO_4$. The substance was also soluble in glacial acetic acid.

Test-tube tests. The results will be tabulated:

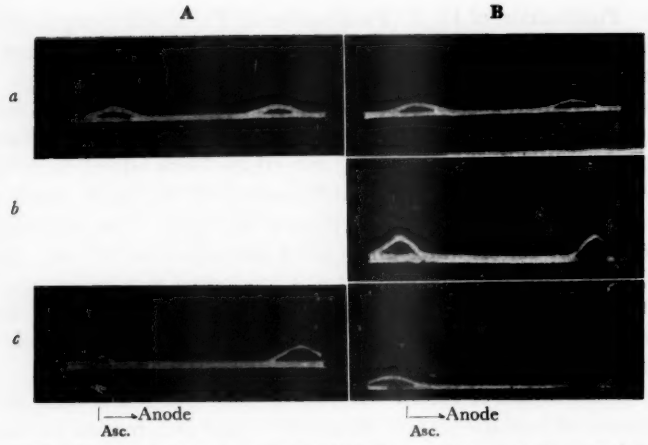

Fig. 1. Electrophoretic patterns (4/5×) of solutions of Prep. I (*a*), II (*b*) and III (*c*) in acetate buffer of pH 4.6 and I 0.2 (**A**) and 0.02 *M* veronal buffer of pH 8.5 whose ionic strength was adjusted to 0.2 with NaCl (**B**). Regarding the concentration of the solutions, see below. Temperature and current 14°C and 8 mA respectively in (**A**) and 16°C and 8.5 mA respectively in (**B**). Exposure 60 minutes after starting current. Mobility:

Prep. I (1%, pH 4.6) $\frac{(-0.46)\times 0.0056\times 0.3}{3600\times 0.0080} = -2.7\times 10^{-5}$ cm²/volt/sec

(0.5%, pH 8.5) $\frac{(-0.22)\times 0.0154\times 0.3}{3600\times 0.0085} = -3.5\times 10^{-5}$,,

Prep. II (0.4%, pH 8.5) $\frac{(-0.26)\times 0.0154\times 0.3}{3600\times 0.0085} = -4.1\times 10^{-5}$,,

Prep. III (1%, pH 4.6) $\frac{(-0.06)\times 0.0056\times 0.3}{3600\times 0.0080} = -0.4\times 10^{-5}$,,

(0.6%, pH 8.5) $\frac{(-0.2)\times 0.0154\times 0.3}{3600\times 0.0085} = -3.2\times 10^{-5}$,,

Test	Preparation		
	I	II	III
Molisch	++	+++	++
Biuret	±	+	+++
Neuberg-Saneyoshi	−	−	−
Osaki-Turumi			
Direct	−	−	−
Indirect	+	+	+
Phosphorus	−	−	−
Hydrolysable sulfur	+	−	−

Assay by paper partition chromatography. a) Sugar components. Masamune and Yosizawa's procedure[3] was adapted. 5 mg. each of Preps.

I and II and 6 mg. of III were hydrolyzed with 0.5 cc. of 1 *N* H_2SO_4, and the hydrolysates, freed from excess H_2SO_4 and distilled to dryness, were dissolved in 0.1 cc. of water, and 0.05 cc. portions were subjected to the descending chromatography and indicated according to Partridge. As shown in Fig. 2, galactosamine, glucosamine, galactose, glucose, mannose, L-fucose and xylose and/or lyxose were detected in any of the preparations.

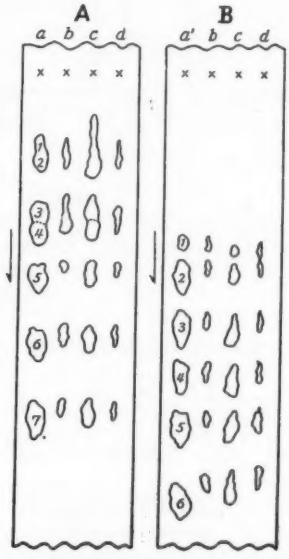

Fig. 2. Sugar chromatograms developed by descending irrigation with *n*-butylacetate-acetic acid-ethanol-water (3:2:1:1 by volume) (**A**) and butanol-pyridine-water (5:3:2 by volume) (**B**) of the hydrolysates of Prep. I (*b*), II (*c*) and III (*d*). Paper slivers Toyo Roshi filter paper No. 2, 60× 10 cm. Temperature and duration of test 22–24°C and 24 hrs. respectively. Indicator the aniline hydrogen phthalate reagent of Partridge.

a, *a'*: reference run. 1 galactosamine, 2 glucosamine, 3 galactose, 4 glucose, 5 mannose, 6 xylose, 7 L-fucose.

b) Amino acid components. 2 mg. each of the substances were heated with 2 cc. of 6 *N* HCl at 100°C for 24 hours in a sealed tube. The hydrolysates were freed from the hydrochloric acid in a desiccator containing solid caustic soda in the manner usual in this Institute (In cases of Preps. II and III, the hydrolysate was centrifuged to remove humin in advance). Each of the evaporated residues was dissolved in 0.1 cc. of water, and the whole (in cases of Preps. I and II) or a half (in the case

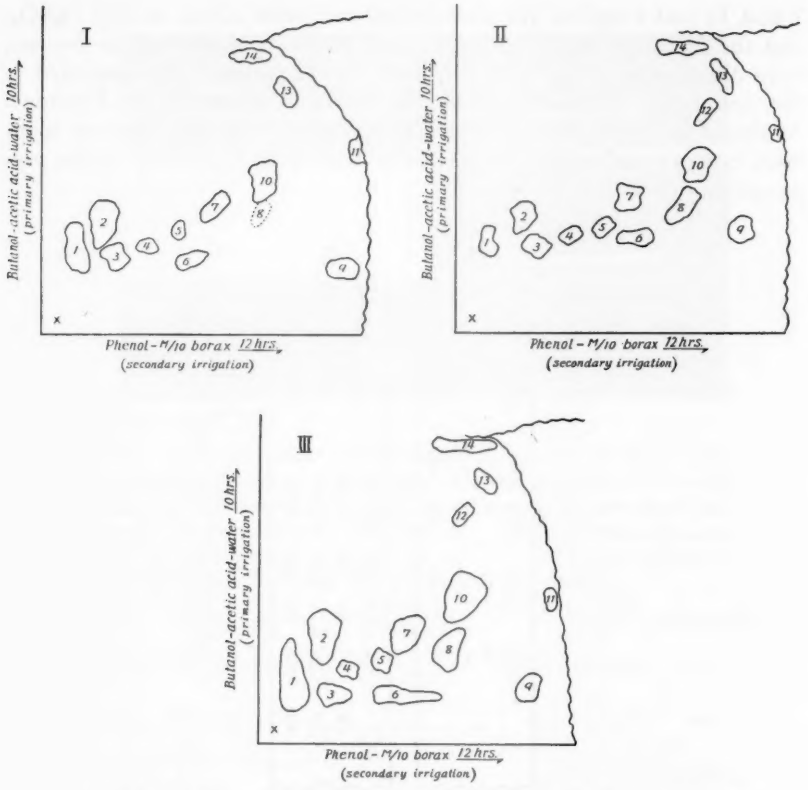

Fig. 3 (I, II, III). Amino acid chromatograms of the hydrolysates of Prep. I (I), II (II) and III (III). Paper squares: Toyo Roshi filter paper No. 2, 30×30 cm. Temperature of test 22–24°C. Indicator 0.1% ninhydrin in a mixture of glacial acetic acid and ethanol in the volume proportion of 3:7.

1 aspartic acid, 2 glutamic acid, 3 cystine, 4 serine, 5 glycine, 6 lysine, 7 threonine, 8 hexosamine, 9 arginine, 10 alanine, 11 proline, 12 tyrosine, 13 valine and/or methionine, 14 leucine and/or isoleucine.

of Prep. III) of the solution was paper-chromatographed after Masamune and Hakomori.[4] As shown in Fig. 3, aspartic and glutamic acids, cystine, serine, glycine, lysine, threonine, arginine, alanine, proline, valine and/or methionine and leucine and/or isoleucine were detected in all the substances and tyrosine besides in Preps. II and III.

Quantitative analysis. The analytical figures are embodied in Table I,

TABLE I

Composition of Preps. I, II and III

Analysis	In per cent			In equivalents per equivalent weight		
	Prep. I	II	III	Prep. I	II	III
N*	8.6	7.9	11.5			
Hexosamine†	3.1	14.3	6.1	1.0	1.0	1.0
Galactose‡	1.4	2.7	0.9	0.46	0.19	0.15
Glucose‡	7.8	7.0	0.9	2.56	0.49	0.15
Mannose‡	2.1	8.9	3.8	0.68	0.63	0.63
L-Fucose§	0.7	2.1	0.8	0.21	0.13	0.12
Xylose and/or lyxose as xylose//	3.9	2.6	1.1	1.06	0.14	0.15
Sialic acid¶	0.9		1.0	0.60		0.34
Ash**	12.4	2.4	1.5			

* Micro Kjeldahl. † Masamune and Yosizawa.[5] ‡ Masamune and Sakamoto.[6] § Masamune and Sakamoto.[7] // Masamune and Sakamoto.[8] ¶ Werner and Odin (by the direct Ehrlich reaction)[9]; Calcd. as $C_{13}H_{23}O_{11}N$. ** Pregl method without use of H_2SO_4.

which indicate that the substances all belong to the category of glucidamins.

Optical rotation in water.

$$\text{Prep. I:}\quad [\alpha]_D^{20}=\frac{-0.323\times 1}{0.0101\times 1}=-32.0^\circ$$

$$\text{Prep. II:}\quad [\alpha]_D^{20}=\frac{-0.339\times 1}{0.0101\times 1}=-33.6^\circ$$

$$\text{Prep. III:}\quad [\alpha]_D^{20}=\frac{-0.768\times 1}{0.0102\times 1}=-75.3^\circ$$

SUMMARY

1. Three glucidamins, insoluble (I) and soluble (II, III) in 90% phenol, were isolated from tumor knods on the rat omentum majus of Yoshida sarcoma in an electrophoretically pure or almost pure state.

2. The glucidamins were found to contain galactosamine, glucosamine, galactose, glucose, mannose, L-fucose and xylose and/or lyxose as sugar components in molecular proportions and amounts different from one glucidamin to another. I was comparatively rich in glucose and pentose, II in hexosamine, glucose and mannose and III in hexosamine and mannose.

3. I contained aspartic and glutamic acids, cystine, serine, glycine, lysine, threonine, arginine, alanine, proline and valine and/or methionine as amino acids, and II and III tyrosine besides those amino acids.

4. Other properties, physical and chemical, of the glucidamins were also examined.

Many thanks are due to Dr. H. Sato in the Pharmacological Institution, Tokyo, who kindly gave the sarcoma material, for the gift and to Dr. S. Tsuiki in this laboratory for his ardent help throughout this investigation. K. Hasizume.

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References

1) Kerr & Seraidarian, J. Biol. Chem., 1949, **180**, 1203.
2) Masamune, H., Hakomori, Masamune, O. & Takase, Tohoku J. Exp. Med., 1956, **64**, 67.
3) Masamune & Yosizawa, *ibid.*, 1953, **59**, 1.
4) Masamune & Hakomori, *ibid.*, 1956, **64**, 59.
5) Masamune & Yosizawa, *ibid.*, this volume, p. 169.
6) Masamune & Sakamoto, *ibid.*, 1956, **63**, 345.
7) Masamune & Sakamoto, *ibid.*, 1956, **63**, 363.
8) Masamune & Sakamoto, *ibid.*, 1956, **63**, 357.
9) Werner & Odin, Acta soc. med. Upsal., 1952, **57**, 232.

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Studies on the Surgery of Bronchiectasis

I. Bronchiectasis Experimentally Produced in Dog

By

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Divers experiments have been made to produce bronchiectasis in animals by many workers. Recently, Tannenberg and Pinner (J. Thorac. Surg., 1942, **11**, 571), by referring to a voluminous literature and making various kinds of experiments, have extensively studied on the causative relationship between pulmonary atelectasis and bronchiectasis.

We also have undertaken a series of studies with a view to clarifying the cause of bronchiectasis. The present paper is to report some interesting information obtained from experiments with dogs.

Experimental

Methods

The following four series of experiments were conducted with dogs:

Series 1. Ligation of the bronchus from without. The chest was opened on the right or left side under intratracheal anesthesia; the main bronchus exposed and ligated with a silk thread below the bifurcation; penicillin scattered in the thoracic cavity and the thoracic wall closed primarily.

Series 2. Introduction of a foreign body into the bronchus of one lung or one lobe. A sponge ball was introduced into bronchus of one lung or one lobe, under local anesthesia through a transverse intercartilaginous tracheotomy to occlude the bronchus.

Series 3. Resection of the unilateral vagus nerve at the cervix. Incision was made on the lateral side of the cervix under local anesthesia, the vagus nerve exposed and resected, and the skin sutured primarily.

Series 4. Valvular incomplete obstruction of the bronchus. An attempt was made to devise a method of valvular incomplete obstruction of the bronchus for producing a high internal pressure in the peripheral

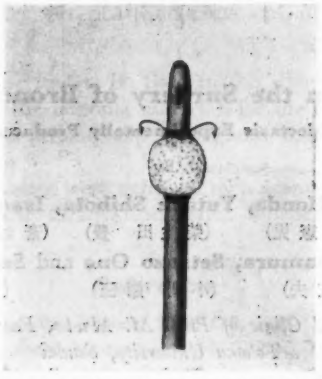

Fig. 1. A devise of valvular incomplete bronchial obstruction in which rubber sponge and fishhook are fixed near the distal end of the Nelaton's catheter

bronchi. A piece of refined rubber sponge was fixed round a slender Nélaton's catheter near the distal end (Fig. 1); a fishhook was fixed between the wall of the catheter and the sponge so as to prevent coughing-up of the foreign body. This special "obstructor" was introduced into the bronchus through a tracheal incision, the Nélaton's catheter fixed hypodermically. Though the dog was attacked by a violent fit of coughing due to the introduction of the obstructor, the fishhook prevented backward movement of the foreign body by piercing the bronchial wall. As the obstructor was made of rubber sponge, its contact with the bronchial wall became so air-tight at the time of expiration as to prevent escape of the air in the alveoli and bronchi, while at the time of inspiration air was allowed to flow into the lung through a small space formed between the sponge and the bronchial wall with dilatation of bronchi. The obstructor devised by us thus served, so to speak, as a valve, highly raising the pressure within the bronchi and alveoli.

At an appropriate time after the operation, as judged from the conditions of the operated dog, antibiotics were introduced through the Nélaton's catheter after drawing off some air from within, with a view to producing a chronic inflammation without acute infection of the bronchi.

In a parallel series of dogs resection of the vagus nerve was performed on the obstructed side.

Results

In series 1 (7 cases of ligation of the bronchus from without) and in series 2 (7 cases of introduction of a foreign body into the bronchus),

bronchiectasis was produced in no cases. In the former series there was seen nothing but atelectasis; in the latter series it was very difficult to fix the foreign body in place, the foreign body being always ejected by coughing. In short, these two series of experiments proved to be unsuccessful (Table I).

TABLE I

Summarized Results of Four Series of Experiments Undertaken to Produce Bronchiectasis

	Unsuccessful	Successful
Ligature of bronchus	7	0
Introduction of foreign body	7	0
Resection of vagus nerve	5	0
Valvular incomplete obstruction — Vagus nerve not resected	10	1
Valvular incomplete obstruction — Vagus nerve resected	11	12

In series 3 (5 cases of vagus nerve resection), changes of the bronchial wall after the operation were examined by means of a bronchoscope everyday, with the results as typically shown in Tables II and III. On the

TABLE II

Changes as Seen in Bronchus after Resection of Vagus Nerve

		Before operation	After operation (days)								
			1	2	4	5	7	13	17	19	21
Not resected side	Cough	++	++	++	++	++	++	++	++	++	++
	Constrictive movement of bronchus and bronchioli	++	++	++	++	++	++	++	++	++	+
	Secretion — Mucous	−	−	−	−	−	−	−	−	−	−
	Secretion — Pussy	−	−	−	−	−	−	−	−	−	−
	Dilatation of bronchus	−	−	−	−	−	−	−	−	−	−
Resected side	Cough	++	++	+	+	+	−	−	−	+	+
	Constrictive movement of bronchus and bronchioli	++	++	+	−	−	−	−	+	+	+
	Secretion — Mucous	−	++	−	−	−	−	−	+	+	−
	Secretion — Pussy	−	−	+	++	++	##	##	++	+	−
	Dilatation of bronchus	−	−	−	+	+	+	+	+	±	±

second day after the operation, the cough reflex and bronchial constrictive movement on the resected side became inactive, and secretion became active. On the 4th to 5th day, the cough reflex and the constrictive movement became invisible or extremely weak, and secretions became

TABLE III

Changes as Seen in Bronchus after Resection of Vagus Nerve

		Before operation	After operation (days)								
			1	2	4	5	7	8	14	16	18
Not resected side	Cough	++	++	++	++	++	++	++	++	++	++
	Constrictive movement of bronchus and bronchioli	++	++	++	++	++	++	++	++	++	++
	Secretion { Mucous	−	−	+	−	−	−	−	−	−	−
	Secretion { Pussy	−	−	−	−	−	−	−	−	−	−
	Dilatation of bronchus	−	−	−	−	−	−	−	−	−	−
Resected side	Cough	++	++	++	++	+	+	+	+	++	++
	Constrictive movement of bronchus and bronchioli	++	++	++	++	+	+	+	++	++	++
	Secretion { Mucous	−	−	+	+	++	++	++	++	−	−
	Secretion { Pussy	−	−	−	−	−	+	+	+	−	−
	Dilatation of bronchus	−	−	−	+	+	+	+	±	±	−

pussy; as compared with the bronchus on the normal side, the bronchus on the operated side was found to have become expanded, revealing a paralytic dilatation. These changes could be seen for 8 to 14 days after the operation, then gradually became less and less until the preoperative conditions were recovered 10 to 19 days after the operation. Thus, mere resection of the vagus nerve brought about no other changes than a temporary increase of intrabronchial secretion and a temporary diminution of bronchial constrictive movement; an attempt to produce bronchiectasis was unsuccessful.

In series 4 (44 cases of valvular incomplete obstruction of the bronchus), no typical bronchiectasis could be produced in 10 of 11 cases in which resection of the vagus nerve on the obstructed side had not been performed, while in the remaining 1 case, in which the dog survived for three months after the operation, the experiment was successful in producing bronchiectasis, as shown in Fig. 2. From a comparision of the postoperative picture (b) with the preoperative one (a) it will be understood that the bronchus on the obstructed side became abnormally expanded.

Of the 23 cases in which both valvular incomplete obstruction and resection of the vagus nerve had been conducted, 12 cases revealed remarkable bronchial dilatation as typically shown in Fig. 3 to 6.

DISCUSSION

Many and various experiments have been undertaken to elucidate

Fig. 2, a

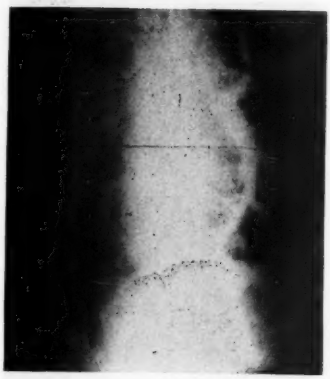

Fig. 2, b

Fig. 2, a. Bronchogram before operation.
Fig. 2, b. Bronchogram after operation.

Fig. 3

Fig. 4

Fig. 3. A case of the experimentally produced bronchiectasis.
Fig. 4. A case of the experimentally produced bronchiectasis.

the origin of bronchiectasis by the use of laboratory animals, mostly of rabbits. In our laboratory, dogs have been used in this line of study. Bronchial ligation, which was performed as the first trial, was not successful in that the ligation could not cause bronchiectasis but only atelectasis. Tannenberg and Pinner also state that it is difficult to produce bron-

Fig. 5

Fig. 6

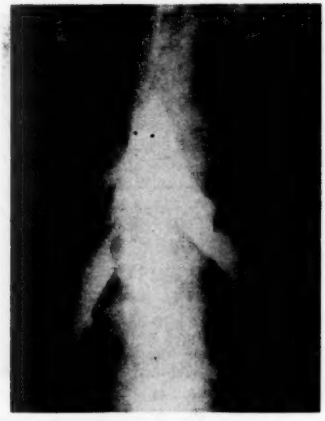

Fig. 5. A case of the experimentally produced bronchiectasis.
Fig. 6. A case of the experimentally produced bronchiectasis.

chiectasis by mere ligation, and infection that produces a purulent inflammation of the bronchi is necessary for this.

As a second trial, a foreign body was introduced into the bronchus. It was impossible, however, to keep the introduced body in place, the body always being coughed out, and this trial also was unsuccessful.

With a view to settling these difficulties (i.e., production of mere atelectasis by complete bronchial obstruction and ready coughing-out of a foreign body introduced), a device for valvular incomplete obstruction was made so as to hold the " obstructor " long in place and cause an incomplete obstruction. Even with this device, however, it was very difficult to produce bronchiectasis, and it seemed necessary to take into account some other factors too.

During the course of study we came across a patient of bronchiectasis accompanying Raynaud's disease. Being suggested by this that there may be some correlation between bronchiectasis and some disorder in the autonomic nervous system, we made a test of resecting the vagus nerve, with the results that 1) mere denervation was incapable of producing bronchiectasis, but 2) a homolateral valvular incomplete obstruction performed in combination with the denervation was capable of causing this disease readily. Thus, it may well be concluded that some disorder in the autonomic nervous system takes a part in producing this disease.

Summary

With a view to elucidating the origin of bronchiectasis, four series

of experiments were performed. When valvular incomplete obstruction was combined with the homolateral resection of the vagus nerve, bronchiectasis was readily produced.

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Studies on the Surgery of Bronchiectasis

II. Histologic Study of Changes in the Mucous Membrane of Bronchus

By

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The literature on histologic study of bronchiectasis is abundant, and especially as to chronic inflammation of bronchi, full information is available. In the course of histopathologic studies on this disease, examining serial sections prepared from resected specimens, we found changes indicative of precancerous degeneration in 4 of the 10 cases examined and could obtain several pictures showing varying extents of malignancy, as will be reported in the following.

The age and lesions of patients from whom serial sections were prepared are shown in Table I. The following findings were common to all of the 10 cases studied; 1) an uniform chronic inflammation of bronchial mucosa from bronchi to alveoli; 2) some extent of epithelial hypertrophy and hyperplasia. Most of the pictures of the hypertrophy and hyperplasia seen in the present study represented papillary proliferation toward the bronchial lumen or dendritic invasion into the submucous tissue.

Fig. 1 shows papillary proliferation of several layers of tall cylindrical cells in more proximal parts of respiratory bronchioles; Fig. 2 represents papillary proliferation of low cylindrical cells toward the lumen in more distal parts of respiratory bronchioles; and Fig. 3 shows several layers of low cylindrical cells dendritically invading the submucous tissue in distal parts of respiratory bronchioles.

In some cases, pictures showing more advanced stages of hyperplasia were seen. Thus, in Okuyama's case (Fig. 4, bronchogram; Fig. 5, resected lung) there were observed a hyperplasia toward the lumen at the seemingly transitive parts between respiratory bronchioles and alveolar passages. The hyperplasia was made up of a few layers of cuboidal cells

TABLE I
Age and Lesions of Patients

Patient			Lesion	Operation
Name	Age	Sex		
Igari	19	Male	R. lung	R. pneumonectomy
Murakami	20	Male	L. lung	L. pneumonectomy
Endo	23	Female	R. lower lobe	R. lobectomy
Sato	24	Male	R. lung	R. pneumonectomy
Okuyama	30	Female	L. lung	L. pneumonectomy
Isago	30	Male	Both lower lobes	L. lobectomy
Miura	33	Male	Both lower lobes	L. lobectomy
Chiba	36	Male	L. lung	L. pneumonectomy
Kishi	46	Male	L. lower lobe	L. lobectomy
Endo	48	Female	L. lower lobe	L. lobectomy

with hyperchromatic nuclei and a small amount of interstitial tissue (Fig. 6).

In Endo's case (Fig. 7, bronchogram; Fig. 8, resected lung) it was noted at alveolar passages that cuboidal cells, with hyperchromatic nuclei and unaccompanied by interstitial tissue, made a papillary proliferation toward the lumen, as shown in Fig. 9. In some respiratory bronchioles, several layers of cuboidal cells with pale cytoplasm and round chromophobic nuclei showed particulary active proliferation, and these cells superposed like a stone wall and projected into the lumen (Fig. 10).

From these findings obtained with Okuyama's and Endo's cases it is clear that the hyperplasia in these cases had advanced beyond those stages such as shown in the first 3 pictures (Figs. 1 to 3) toward precancerous degeneration, but at the same time it cannot be asserted that the changes had become precancerous. We obtained, however, the following 2 cases, which gave pictures indicating undoubtedly precancerous degeneration:

Chiba's case (Fig. 11, bronchogram; Fig. 12, resected lung): There were found, in respiratory bronchioles, cell nests of hyperplastic epithelium being formed insularly in the lumen as shown in Fig. 13. The cells in outer part of these nests were cylindrical and the cells in the centers of these nests were cuboidal containing hyperchromatic nuclei. No basement membrane was perceived in these nests. These nests seem to have been formed by very active growth of the hyperplastic epithelium of the mucous membrane. It was also found in parts of respiratory bronchioles that the hyperplastic epithelium of the mucous membrane partly formed cell nests in the submucous tissue, as shown in Fig. 14. As can be seen from a picture of high magnification of one of these areas (Fig. 15), many

cells were polymorphous, not clearly outlined, and their nuclei were large, round or oval, and chromophobic. The basement membrane became rough and showed a tendency to destruction. A mitotic figure was found in the cell nests. These findings may be asserted to represent precancerous change.

Another case of precancerous degeneration was observed: In Igari's case (Fig. 16, bronchogram: Fig. 17, resected lung), solid cell nests were found to have been formed by epithelial cells of the mucous membrane in some areas of respiratory bronchioles or alveolar passages. The cells of the nests were cuboidal and had hyperchromatic nuclei, as shown in Fig. 18.

Discussion

Cases in which carcinoma of the lung developed from bronchiectasis have been reported by several authors[1),2)] including Bass and Singer,[3)] Löhlein,[4)] Womack and Graham,[5)] and Siegmund.[6)] We[7)] too have communicated a combined case of bronchiectasis and pulmonary carcinoma, in which it was ascertained that the two diseases are very closely related with each other.

Microscopic examination of serial sections prepared from 10 resected lungs of our 10 cases of bronchiectasis gave the following findings: In the cases of Okuyama, Endo, Chiba and Igari there were obtained histologic pictures indicating active epithelial proliferation in the mucous membrane of the bronchioli; in the first 2 cases pictures revealing a proliferation near a precancerous state were found, while the tendency to malignancy on the part of epithelium of the mucous was much stronger in the last 2 cases than in the first 2. Thus, in Chiba's case a mitotic figure was recognized, and in Igari's case the occurrence of precancerous change was demonstrated in that atypically proliferated cells with hyperchromatic nuclei formed solid cell nests.

According to Aschoff,[8)] primary bronchial carcinoma is liable to originate in respiratory bronchioles (the first to third orders). It is to be noted in our cases that precancerous changes were often seen in the transitive parts between the respiratory bronchioles and the alveolar passages. Siegmund etc.[4),6)] state that pulmonary carcinoma is produced by epithelial metaplasia of the mucous membrane, while our findings suggest the possibility that pulmonary carcinoma takes its origin from epithelial hyperplasia of the mucous membrane.

The fact obtained by our researching that the precancerous changes were not rare in bronchiectasis may be considered to furnish a basis for an early operation.

SUMMARY

Microscopic examination was made of serial sections from 10 resected lungs to study changes in the epithelium of the mucous membrane, with the results that precancerous changes were recognized in 4 of the 10 cases.

References

1) Hart & Mayer, Henke-Lubarsch's Handb. spez. path. Anat. u. Hist., Berlin, III-1, 1928, p. 498.
2) Kaufmann, Lehrbuch der spez. path. Anat., Berlin & Leipzig, I, 1931, p. 438.
3) Bass & Singer, Ann. Int. Med., 1951, **34**, 498.
4) Löhlein, Verhandlungen der deutschen pathologischen Gesellschaft, 1908, **12**, 111.
5) Womack & Graham, Am. J. Pathology, 1941, **17**, 645.
6) Siegmund, Virchow's Arch, 1922, **236**, 191.
7) Honda, Unpublished.
8) Aschoff, Pathologische Anatomie, Jena, II, 1936, p. 289.

Fig. 1

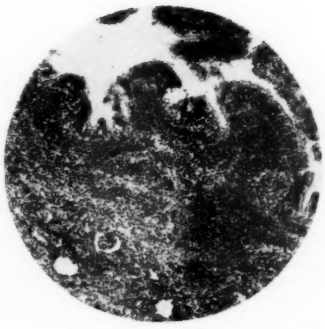

Fig. 2

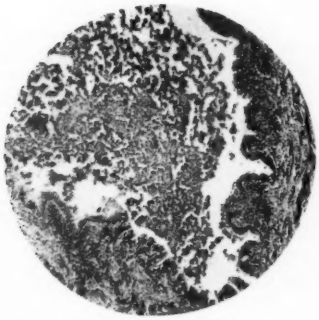

Fig. 1. Papillary proliferation of several layers of tall cylindrical cells in more proximal parts of respiratory bronchioles.

Fig. 2. Papillary proliferation of low cylindrical cells toward the lumen in more distal parts of respiratory bronchioles.

Fig. 3

Fig. 4

Fig. 3. Several layers of low cylindrical cells dendritically invading the submucous tissue in distal parts of respiratory bronchioles.

Fig. 4. Bronchogram of Okuyama's case.

Eig. 5

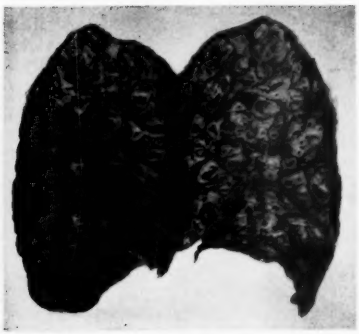

Fig. 6

Fig. 5. Resected lung of Okuyama's case.

Fig. 6. Microscopic picture of Okuyama's case. There were observed hyperplasia of epithelium toward the lumen at the seemingly transitive parts between respiratory bronchioles and alveolar passages.

Fig. 7

Fig. 8

Fig. 7. Bronchogram of Endo's case.

Fig. 8. Resected lung of Endo's case.

Fig. 9

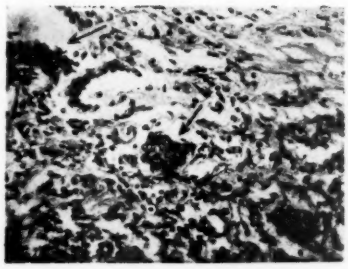

Fig. 10

Fig. 9. Microscopic picture of Endo's case. It was noted at alveolar passages that cuboidal cells, with hyperchromatic nuclei and unaccompanied by interstitial tissue, made a papillary proliferation toward the lumen.

Fig. 10. Microscopic picture of Endo's case. Several layers of cuboidal cells with pale cytoplasm and round chromophobic nuclei showed particulary active proliferation.

Fig. 11

Fig. 12

Fig. 11. Bronchogram of Chiba's case.

Fig. 12. Resected lung of Chiba's case.

Fig. 13

Fig. 14

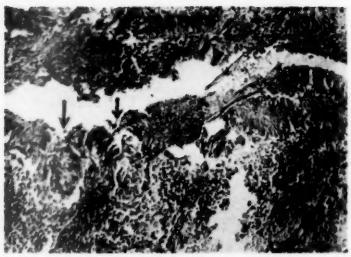

Fig. 13. Microscopic picture of Chiba's case. Cell nests of hyperplastic epithelium being formed insularly in the lumen of respiratory bronchioles.

Fig. 14. Microscopic picture of Chiba's case. The hyperplastic epithelium of mucous membrane partly formed cell nests in the mucous tissue.

Fig. 15

Fig. 16

Fig. 15. Microscopic picture of Chiba's case. Many cells were polymorphous, not clearly outlined, and their nuclei were large, round or oval, and chromophobic. A mitotic figure was found in the cell nests.

Fig. 16. Bronchogram of Igari's case.

Fig. 17

Fig. 18

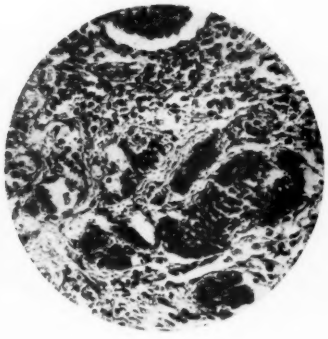

Fig. 17. Resected lung of Igari's case.

Fig. 18. Microscopic picture of Igari's case. Solid cell nests in some areas of respiratory bronchioles or alveolar passages. The cells of the nests were cuboidal and had hyperchromatic nuclei.

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On the Ether Concentration of Inspired and Expired Air during Endotracheal Anesthesia

By

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There have appeared in Western countries a number of reports on the measurement of the ether concentration of inspired air during endotracheal anesthesia[1)-5)], while the literature on this problem is scanty in our country. We have already reported[6),7)] on changes in ether concentration of inspired and expired air as measured by means of the Orzat's apparatus. The purpose of the present paper is to add those cases which have accumulated thereafter, and also to describe slight modifications made to the original method.[8)]

Apparatus. The Orzat's apparatus, made by the Glass Institute of our university, was used for gas analysis. Essentially the apparatus consists of a levelling cylinder N, a gas burette B, a stopcock A for gas outlet, absorption tubes I-III, and a stopcock h for introducing expired or inspired gas (Fig. 1). Instead of water as used in the original method, mercury was used to fill the gas burette, for water absorbs ether to some extent. To lessen the weight the burette with a volume of 50 cc was used, which was graduated in scale divisions of 1 cc. As in the original method, 50 per cent potassium hydroxide solution was used to absorb CO_2, 25 per cent pyrogallol-alkali solution to absorb O_2, and concentrated sulfuric acid solution to absorb ether.[9)]

Procedure. With stopcock A open, levelling cylinder N is raised to fill gas burette B with mercury, thus expelling the air inside. Stopcock A is closed, h is turned so as to introduce the gas to be tested into B by lowering N. This procedure is repeated 3 or 4 times. When the gas sample has been admitted into B up to mark 0 by adjusting h and N, h is closed. The stopcock of the ether absorber (I) is then opened to introduce the sample into I, where the ether contained in the sample is allowed to be absorbed. The gas is driven to B to take a burette reading,

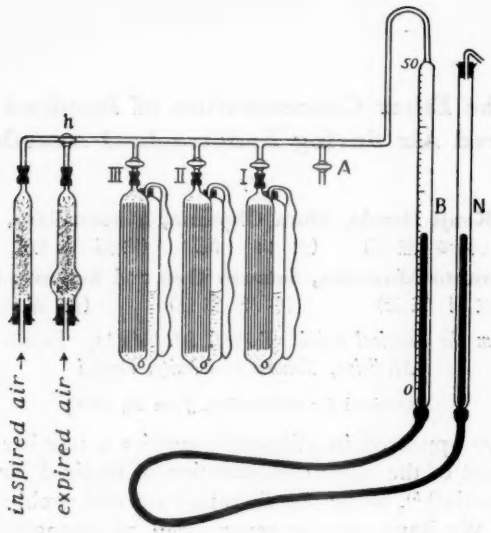

Fig. 1. Respiratory gas analysis apparatus.

from which the volume decrease of the gas can be found. This process is repeated several times until all the ether has been absorbed and a constant reading is obtained. The stopcock of I is now closed and a burette reading is taken to calculate the volume decrease of the gas. The value twice this volume decrease corresponds to the content of ether in volume per cent of the gas sample.

Then, the stopcock of the CO_2 absorber (II) is opened to allow the sample from which ether has been absorbed to flow into II. The same procedure as above is followed and the CO_2 content of the sample is found. The O_2 content is then found by repeating the same procedure as before after opening the stopcock of the O_2 absorber (III).

Check of accuracy of the apparatus. To check the accuracy of this apparatus, measurement was made with samples of known ether concentration. For obtaining ether gas of known concentration an ether vaporization apparatus as shown in Fig. 2 was made, with which saturated ether gas was produced at a given temperature. Fig. 3 represents a vapor pressure curve for ether gas in a text book,[10] and Fig. 4 shows a theoretical curve between concentration of saturated ether gas and temperature at an atmospheric pressure of 760 mm. Hg, the value for each concentration being obtained by calculation from the curve in Fig. 3. A large number of points plotted in Fig. 4 represent observed values for ether

concentration at varying temperature. It will be seen that there are little differences between observed and theoretical values, thus showing the satisfactory accuracy of this apparatus.

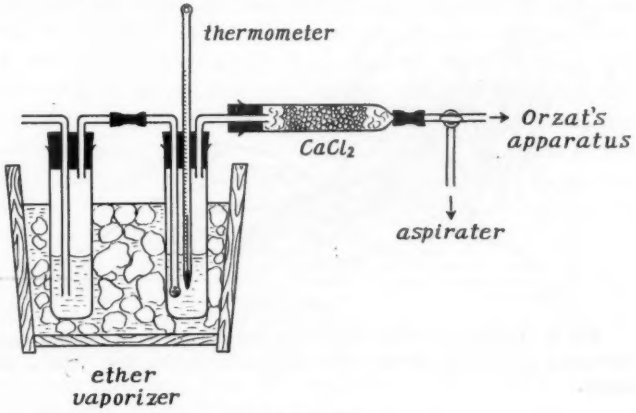

Fig. 2. Ether vaporization apparatus.

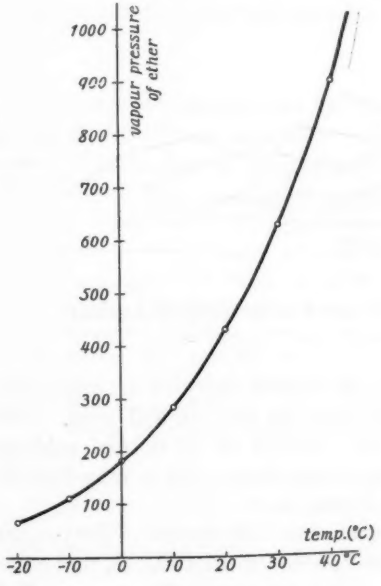

Fig. 3. Vapor pressure curve for ether.

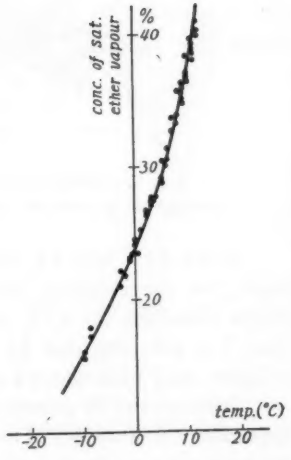

Fig. 4. Concentrations of saturated ether solution at varying temperatures at an atmospheric pressure of 760 mm Hg.

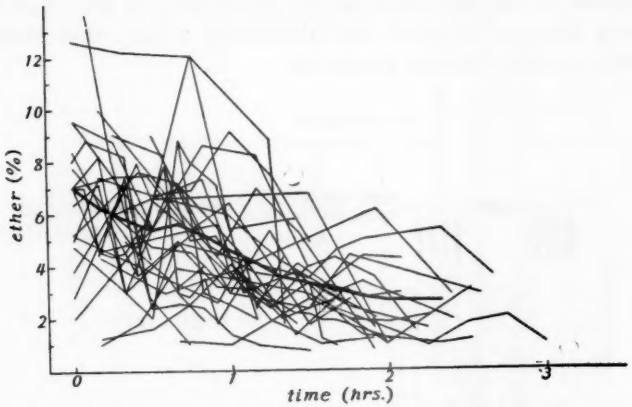

Fig. 5. Changes in ether content of inspired gas in individual cases (light-lined curves) and average ether contents at varying time (heavy-lined curve).

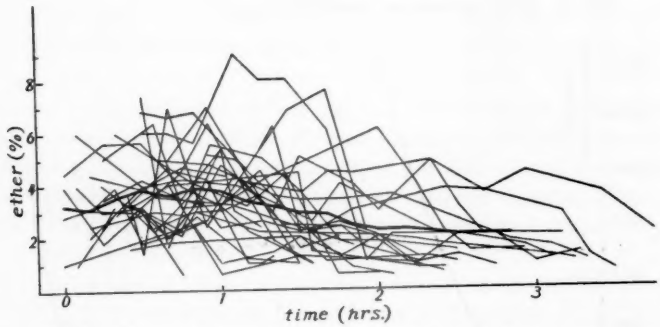

Fig. 6. Changes in ether content of expired gas in individual cases and average ether contents at varying time.

As for CO_2 and O_2 measurement, no special check of accuracy was made, for the methods used were the same as the original ones. The results obtained for CO_2 and O_2 of the expired air of normal subjects were 2–4 per cent and 14–18 per cent, respectively, which may be said to agree well with those found in the literature.

Application of the present method to clinical cases. By the use of the present apparatus the analyses of anesthetic atmosphere for the CO_2, O_2 and ether contents were made during general anesthesia by ether. The cases studied consisted of 17 cases of thoracoplasty, 14 of gastrectomy, 4 of pulmonary lobectomy, 3 of nephrectomy, 2 of amputatio mammae, and 1 of resection

of the lower jaw. In these cases the patients were first subjected to the premedication by atropine or morphine, then to the induction by intravenous injection of pentothal sodium, followed by ether inhalation. Serial examinations of the ether content of expired and inspired gas were made at intervals of 10 to 15 minutes after administration of ether had begun. Figures 5 and 6 show the results obtained with these 41 cases (Fig. 5 for inspired air, Fig. 6 for expired air), where curves in light lines represent changes in ether content of individual cases during the course of anesthesia and a curve in heavy line in each figure shows a variation of the mean values of 41 cases. From Fig. 5 it will be seen that, though there were found wide variations of values according to cases, mean values fell within a range of 5 to 10 per cent in the induction and of 2 to 5 per cent in the maintenance, which results are in general agreement with those thus far reported in the literature. Changes in ether concentration of expired gas were not so great as those of inspired gas, as will be seen from Fig. 6; mean values fell within a range of 2 to 4 per cent.

From these finding it may be concluded that an ether concentration of 5 to 10 per cent (mean: 7 per cent) in the induction and of 2 to 5 per cent (mean: 3 per cent) in the maintenance, which have been reported in the literature,[11] are the concentrations most suitable for Japanese also.

Conclusion

The ether concentration of inspired and expired air in 41 cases was measured by Orzat's apparatus modified by us. The mean value of the ether concentration of expired air in the induction was 7%, that in the maintenance 3%.

References

1) Haggard, J. Biol. Chem., 1923, **55**, 131.
2) Shaffer & Ronzoni, J. Biol. Chem., 1923, **57**, 741.
3) Faulconer & Ridley,, Anesthesiology, 1950, **11**, 265.
4) Strayer, *ibid.*, 1950, **11**, 599.
5) Ridley, Faulconer & Osborn, *ibid.*, 1951, **12**, 276.
6) Muto, Geka no Ryoeki (Jap.), 1953, **1**, 5.
7) Honda, Sato, Endo, Tomisawa & Suzumori, Nippon Gekagakkai Zassi (Jap.), 1952, **53**, 599.
8) Treadwell, Lehrb. anal. Chem., Leipzig u. Wien, II, 1927, p. 703.
9) Schmidt, Lehrbuch Pharm. Chem., Braunschweig, II, **1**, 1922, p. 331.
10) Landolt-Börnstein Physikalisch-chemische Tabellen, Berlin, 1912.
11) Collins, Principles and practice of anesthesiology, Philadelphia, 1952, p. 263.

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Free Amino Acids in the Gastric Juice of Patients with Gastric Cancer

By

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Although it has been pointed out a few investigators[1)-3)] that in the gastric juice of gastric cancer, amino acids will show a remarkable increase over the normal level, but their details are not clear. Therefore, free amino acids of gastric juice in surgical gastric diseases were examined, as reported herein, using the method of paper chromatography developed by Consden, Gordon and Martin.[4)]

Experimental

Material and Method

To collect pure gastric juice free from food rests, the stomach was cleansed during the night preceding examination, and early in the next morning the Lyon's duodenal catheter was introduced into the empty stomach and the remaining gastric juice was aspirated as completely as possible, and after 20 minutes or more the gastric juice to be analyzed was collected.

Mucus was removed by filtering the collected gastric juice. After adding thymol, the filtrate was deproteinized with alcohol according to the method of Sawazaki and others.[5)] The supernatant fluid was transferred to an evaporating dish and evaporated to dryness at 50 to 60°C. The dried material was dissolved in 5 cc. of distilled water. After adding chloroform the deproteinization procedure was repeated twice. The supernatant fluid was again evaporated to dryness. The dried material thus obtained was dissolved in distilled water at the rate of 100 mg. per cc. of water, and the resulting solution was used for analysis.

Amino acid determinations were made by the two-dimensional ascending method of paper chromatography, using 0.01 cc. of the solution to be analyzed. The filter paper used (Toyo No. 2) was in the form of sheets 23×24 cm. The solvents used for chromatographic development were phenol saturated with water and the *n*-butanol-acetic acid-water

mixture. The development was done in a tightly covered glass vessel, at temperatur from 15 to 22°C with a variation of less than 5°C during a single development.

When the solvent front had advanced about 20 cm., the paper was removed, dried over an electric heater, and sprayed with a 0.25 % ninhydrin solution in saturated butanol-water. After placing the paper in an oven at 80 to 100°C for 15 minutes to reveal amino acid spots, the results of the chromatogram were judged.

The amino acids were identified by their color tints, Rf values and relative positions on the paper, and by checking with a reference chromatogram prepared with 0.01 cc. of a mixture of 12 known amino acids: leucine, phenylalanine, valine, tyrosine, alanine, arginine, lysine, glucosamine, glycine, glutamic acid, aspartic acid and cystine. Determination of other amino acids was made by referring to previously published data.[6),7)]

It was often the case with certain amino acids that some differences were noticed in color intensity of the spots between chromatograms developed first with phenol and those first with butanol; in such cases the final decision was made by comparing both chromatograms.

The color intensity of the spots on amino acid maps is represented here by using the following five grades which have been defined arbitrarily:

−, negative, representing that no color spot was revealed.
±, very weakly positive, representing that the spot could hardly be discerned by reflected light, being visible only by transmitted light.
+, weakly positive, representing that the spot could be discerned by reflected light.
++, moderately positive, representing that the spot was manifest.
+++, strongly positive, representing that the spot was very intense in color.

This method or representation may serve as a rough relative measure of the amount of each amino acid, for a spot of more intense color is obtained when an increased amount of amino acid is run on a chromatogram.

No other special quantitative method was used in the present investigation.

Results

1) Free amino acids in saliva

Examination was made on the free amino acids in the saliva of 3 normal individuals and of 4 admitted patients by the same method as used for gastric juice, with the results as shown in Table I that leucine, valine and alanine were weakly positive in 1 or 2 cases respectively, other amino acids being either very weakly positive or negative.

TABLE I

Free Amino Acids in Saliva

The figure denotes the number of cases

Amino acids	Color intensity of spots			Total spots
	+	±	−	
Leucine	1	2	4	7
Phenylalanine	0	0	7	7
Valine	2	4	1	7
Tyrosine	0	0	7	7
Alanine	2	4	1	7
Threonine	0	0	7	7
Arginine	0	3	4	7
Lysine	0	1	6	7
Histidine	0	0	7	7
Glucosamine	0	0	7	7
Serine	0	3	4	7
Glycine	0	3	4	7
Glutamic acid	0	4	3	7
Aspartic acid	0	3	4	7

2) Free amino acids in the gastric juice of patients suffering from diseases other than gastric cancer.

Seven cases with non-gastric diseases and a total of 12 ulcer cases (6 gastric, 3 duodenal, and 3 complicated gastric and duodenal) were examined for free amino acids of gastric juice, with the results as shown in Table II. In the gastric juice of non-gastric patients leucine, valine, tyrosine, alanine, glucosamine, serine and glycine were moderately positive in 1 to 3 cases, and all of them weakly positive in 3 cases. Other amino acids also were weakly positive in 1 to 3 cases, but threonine and histidine were negative in all the cases.

In the 12 ulcer cases also leucine, valine and alanine were moderately positive in 3 or 4 cases, and weakly positive in 1 to 7 cases, while threonine and histidine were negative in all the cases, and glucosamine was also negative in the majority of the cases.

In the ulcer cases, furthermore, examination was made to ascertain if there exists any correlation of gastric acidity as well as accumulation of gastric contents due to pyloric stenosis with the rate and extent of appearance of individual amino acids. The cases investigated were classed into two groups, of normal acidity and of hyperacidity, the great majority of cases in which one of amino acids were moderately positive (all the 3 cases of leucine, 2 out of 3 cases of valine, and 3 out of 4 cases of alanine)

TABLE II

Free Amino Acids in the Gastric Juice of Cases with Non-gastric Cancer Diseases

The figure in parentheses denotes the number of cases with normal acidity

Amino acids	Non-gastric diseases					Gastric and duodenal ulcer				
	Color intensity of spots				Total spots	Color intensity of spots				Total spots
	++	+	±	−		++	+	±	−	
Leucine	2	3	2	0	7	3 (3)	7	2	0	12 (4)
Phenylalanine	0	1	2	4	7	0	3	3	6	12
Valine	3	3	1	0	7	3 (2)	7	1	1	12 (4)
Tyrosine	1	3	3	0	7	0	1	8	3	12
Alanine	2	3	2	0	7	4 (3)	5	3	0	12 (4)
Threonine	0	0	0	7	7	0	0	0	12	12
Arginine	0	0	3	4	7	0	5	4	3	12
Lysine	0	1	1	5	7	0	5	3	4	12
Histidine	0	0	0	7	7	0	0	0	12	12
Glucosamine	1	1	1	4	7	0	1	1	10	12
Serine	3	1	3	0	7	0	5	4	3	12
Glycine	3	1	3	0	7	0	5	4	3	12
Serine or* Glucosamine	0	0	1	6	7	0	0	0	12	12
Glutamic acid	0	3	3	1	7	0	7	4	1	12
Aspartic acid	0	2	2	3	7	0	4	4	4	12
Basic amino acids*	0	2	1	4	7	0	0	0	12	12

* Indistinguishable.

fell into the group of normal acidity, while a weakly positive cases were distributed almost evenly between the two groups. No special correlation was found between pyloric stenosis and free amino acids.

3) Free amino acids in the gastric juice of patients with gastric cancer.

In 44 cases with gastric cancer free amino acids of gastric juice were examined. It is noteworthy that various amino acids appeared to a considerable extent. In some of these cases, however, it was found that bile had mixed in the gastric juice, and more or less affected the results of amino acid maps. Thus, selecting 28 cases in which gastric juice had been collected in a perfect manner, typical amino acid maps were obtained, their results are shown in Table III.

Attention should be paid to the fact that various amino acids were strongly positive, that is, leucine, valine and alanine were positive in 27 cases (96%). The great majority were moderately or strongly positive.

TABLE III

Free Amino Acids in the Gastric Juice of Patient with Gastric Cancer

The figure in parentheses denotes the number of cases in which the acidity was higher than 21 units.

Amino acids	Color intensity of spots					Total spots
	##	#	+	±	−	
Leucine	19 (6)	6 (2)	2 (1)	0	1 (1)	28 (10)
Phenylalanine	4 (1)	13 (3)	3 (2)	4 (2)	4 (2)	28 (10)
Valine	17 (7)	9 (1)	1 (1)	0	1 (1)	28 (10)
Tyrosine	5 (0)	12 (6)	7 (2)	3 (1)	1 (1)	28 (10)
Alanine	11 (4)	13 (4)	3 (1)	0	1 (1)	28 (10)
Threonine	1 (1)	2 (0)	0	1 (0)	24 (9)	28 (10)
Arginine	7 (3)	13 (4)	3 (0)	1 (0)	4 (3)	28 (10)
Lysine	6 (2)	13 (6)	6 (0)	1 (1)	2 (1)	28 (10)
Histidine	2 (1)	1 (0)	0	1 (0)	24 (9)	28 (10)
Glucosamine	1 (0)	5 (3)	3 (1)	0	19 (6)	28 (10)
Serine	4 (1)	9 (2)	5 (0)	1 (1)	9 (6)	28 (10)
Glycine	4 (1)	9 (2)	5 (0)	1 (1)	9 (6)	28 (10)
Serine or* glucosamine	0	4 (1)	0	0	24 (9)	28 (10)
Serine or glycine*	0	1	0	0	27	28
Glutamic acid	5 (2)	16 (6)	3 (0)	3 (1)	1 (1)	28 (10)
Aspartic acid	3 (1)	16 (3)	3 (0)	4 (1)	2 (1)	28 (10)
Basic amino acids*	0	1 (0)	0	0	27 (10)	28 (10)

* Indistinguishable.

Phenylalanine, tyrosine, arginine, lysine, and glutamic and aspartic acids also were strongly positive in 3 to 7, moderately positive in 12 to 16 and negative only in 1 to 4 cases. Serine and glycine were positive in 4, moderately positive in 9, and negative in 9 cases. Threonine and histidine, which did not appear in cases with ulcer and other diseases, were negative in 24 cases with gastric cancers (84%), but it is to be noted that they were strongly or moderately positive in 1 or 2 of the cases.

In a single case no amino acids were detected on the chromatograms obtained with 0.01 cc. of the test solution (corresponding to 1 mg. of the test material), in which total gastrectomy was performed and cancerous infiltration was noticed all over the wall of the stomach, but no ulcer formation was perceived.

No correlation existed between the locality of cancer and the rate and extent of appearance of amino acids. There appears to be no relation between gastric acidity and the appearance of amino acids, though

examination was made by classfying the cases into two groups, one with the total acidity higher than 21 units, and the other with the acidity lower than 20 units. In gastric cancer cases no high acidity such as seen in ulcer cases took place, but 10 cases in which the total acidity was from 21 to 50 were obtained. The results of examination in these cases are represented in parentheses in Table III.

In 8 of these gastric cancer cases chromatograms were prepared, after the gastrectomy, of the gastric juice free from bile, and definite spots showed in all the cases, with the results as shown in Table IV. It will be seen that

TABLE IV

Free Amino Acids in the Gastric Juice after Gastrectomy

Amino acids	Gastric cancer				Gastric and duodenal ulcer			
	Color intensity of spots			Total spots	Color intensity of spots			Total spots
	+	±	−		+	±	−	
Leucine	2	3	3	8	3	3	0	6
Phenylalanine	0	0	8	8	0	1	5	6
Varine	1	3	4	8	1	4	1	6
Tyrosine	0	1	7	8	1	3	2	6
Alanine	3	2	3	8	2	4	0	6
Threonine	0	0	8	8	0	0	6	6
Arginine	1	1	6	8	1	3	2	6
Lysine	1	1	6	8	0	2	4	6
Histidine	0	0	8	8	0	0	6	6
Glucosamine	1	0	7	8	0	1	5	6
Serine	0	1	7	8	1	1	4	6
Glycine	0	1	7	8	1	1	4	6
Serine or* Glucosamine	0	2	6	8	0	0	6	6
Glutamic acid	1	3	4	8	3	2	1	6
Aspartic acid	0	2	6	8	1	1	4	6
Basic amino acid*	1	1	6	8	0	0	6	6

* Indistinguishable.

many amino acids remarkably decreased after gastrectomy: Leucine, valine, alanine and some other amino acids were either very weakly positive or negative with the exception of 1 to 3 weakly positive cases. In gastric ulcer, decreasing of many amino acids in gastric juice after gastrectomy was also seen. There were only few cases in which amino acids after the gastrectomy were as moderately positive, as before the

operation.

Discussion

From the results obtained by applying the method of determining amino nitrogen it has been pointed out that the free amino acids in gastric juice exceed the normal level in cases with gastric cancer. Changes of individual amino acids, however, are not clear. Cagnianut and others,[8] studying the free amino acids by paper chromatography, have found that glutamic and aspartic acids and polypeptides appeared and basic amino acids increased remarkably in 2 cases of gastric cancer.

By a similar method of analysis it was found that several amino acids appeared notedly in gastric juice of patients with gastric cancer, while in cases with gastric or duodenal ulcer or other diseases their appearance was infrequent and only slight in extent, if any, and that a marked decrease occurred after gastrectomy. Thus the writer regards the marked appearance of free amino acids in gastric juice to be a phenomenon quite peculiar to gastric cancer.

Examination was made to see if there exists any correlation between this marked appearance of amino acids in gastric cancer cases and the decrease of gastric acidity peculiar to gastric cancer. The results show that leucine and some other amino acids were moderately or strongly positive in the 10 cases where the acidity was 21 to 50 units and also in the remaining cases with the acidity lower than 20 units. Thus the change in acidity of such an extent as seen in gastric cancer did not affect the appearance of free amino acids in gastric juice. With ulcer, however, cases moderately positive regarding amino acids were seen more frequently in the group of normal acidity than in that of hyperacidity, though the cases examined were small in number. This problem may deserve future study.

According to Martin,[2] who determined the amino nitrogen in gastric juice, the nitrogen was 7.72 mg/dl on an average in ulcer cases, the value being nearly normal, while in cases with benign achlorhydria the value increased to as much as 12.71 mg/dl on an average, and in cancer cases it was 25.67 mg/dl on an average, a value much higher than in benign achlorhydria. Since every case was taken not to mix saliva in collecting gastric juice, and that the amino acids detected in saliva were very small in amount, it is clear that the remarkable appearance of free amino acids in cases with gastric cancer was not due to saliva mixed in gastric juice.

The free amino acids that were detected most markedly in the gastric juice of gastric cancer patients were leucine, valine and alanine, which appear also in the gastric juice of patients with ulcer and other diseases, though to only a slight degree. Furthermore, phenylalanine, tyrosine,

arginine and lysine were also detected to some extent. Contrary to this, threonine, histidine and glucosamine rarely appeared in gastric cancers and in ulcer and other diseases. According to Cagnianut and others,[8] a small amount of alanine, leucine, histamine and glucosamine were found in normal gastric juice to a minor degree, while glutamic acid and aspartic acid made their appearance in gastric cancers, and basic amino acids increased conspicuously. Thus some differences in kind of amino acids are seen between the results obtained by Cagnianut and others and by the writer. It is not clear, whether this is due to differences in experimental methods and in judgement of the results or to differences in cases.

Summary

Employing the method of paper chromatograph, examination was made on amino acids in gastric juice in cases with gastric cancer, gastric and duodenal ulcers and other diseases. It was found that in gastric cancer cases several amino acids, expecially leucine, valine and alanine, appeared remarkably.

Thanks are due to Dr. Gunji Tomita of the Biological Institute of the Tohoku University for his kind guidance in chromatographic techniques.

References

1) Derrer, Bratislav. Lek. Lysty, 1927, **4**; Cited in Zbl. Chir., 1927, **54**, 2806.
2) Martin, J. Amer. Med. Assoc., 1933, **100**, 1475.
3) Sumida & Hongo, Nagasaki Igk. Z. (Jap.), 1930, **8**, 918.
4) Consden, Gordon & Martin, Biochem. J., 1944, **38**, 224, Cited in Biochem. J., 1948, **43**, 169.
5) Sawazaki, Murakuni, Kagayama & Fukui, Sanka Fuzinka (Jap.), 1951, **17**, 384.
6) Warabioka, Yakugaku (Jap.), 1950, **3**, 115.
7) Dent, Biochem. J., 1948, **43**, 169.
8) Cagianut, Zehnder & Nager, Schweiz. med. Wschr., 1948, **80**, 819.

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The Effect of Menstrual Cycle on Electric Flicker

By

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As is well known, the menstrual cycle is accompanied by cyclic changes of various functions of the body such as physical and chemical properties of the blood,[1),2)] the basal metabolism,[3),5)] the body temperature,[6),8)] the antibacterial agency,[9)] the potential difference of the skin,[10)] etc. In general, women in the menstrual phase are more liable to fatigue than usual; the efficiency of labor is said to decrease during menstruation in 60–80 per cent of female workers.[11),15)] In view of the fact that fatigue of a slight degree can be detected by Motokawa's method of electric flicker,[16),19)] we attempted in the present investigation to find any cyclic change in values of electric flicker which might be correlated with the menstrual cycle.

Experimental

Method

Subjects were 6 healthy students of our training-school for nurses, aged 16 years and having a regular menstrual cycle. As all of them were boarding students of the school dormitory, their conditions of life and business were all alike.

The apparatus used for measuring electric flicker values was the same as in the previous report by Motokawa and Suzuki.[17)] The stimulating electrodes were placed at the outer corners of the eyes of a subject, and the eyes were stimulated with rectangular pulses of 20 cycles per second. The stimulating voltage was increased from zero at a constant rate of 80 mV per second to determine the threshold for the appearance of flicker, S_1, and then the threshold for the disappearance of flicker, S_2, was determined by lowering the voltage from a sufficiently high level at the same rate as above. The value of electric flicker (EFV) is represented by the difference of both sorts of threshold, S_1-S_2. In one series such values were determined 5 times in succession, and averaged. For each subject

measurements extended over two and a half months with one series a day except for a Sunday or a holiday.

Results

During the observation period two or three phases of menstruation were encountered in each subject. The duration of the menstrual phase was 4 days in 9 cases, 3 days in 2 cases and 5 days in one case.

The protocols of the data for 6 subjects are represented in Table I.

TABLE I

Changes of EFV in mV in Menstrual Cycle

	Day before and after menstruation	Subject and Age												Mean value	Standard deviation
		A.T., 16		K.A., 16		A.T., 16		A.H., 16		C.G., 16			T.G., 16				
		Sequence of menstruation															
		1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	3rd	1st	2nd	3rd		
Pre-menstrual phase	10							80						100	180		
	9			140			60	120			200			180	200		
	8			140			120				100						
	7			80			100	160			100	160		80	160		
	6			120				80				120				124.4	54.3
	5	100		120		40	80	100		140	160				180		
	4	170		160		120	60	100		100		120	220	140	160		
	3	160				200		100		80			140	180	180		
	2	160	40	100		60	80	140	40	40	60	100	240	60	200		
	1	80	40	100		240			100	60	80	160	300	140			
Menstrual phase	1	260	120	220		440	260	540	500	240	220	200	320	400	180		
	2		180	240			160	400			200	280	320		400		
	3	280	220	200		400	200	360	400	420	200				340	286.8	114.0
	4	160				440	160	400	340	240		120	500		160		
	5											140					
Post-menstrual phase	1	240		120		100		100	120	180		60	280		200		
	2	120	60			120	30			40		160	160		280		
	3	120	40	100		60	60		140	60		60	220				
	4	110	60	40		100	100	100	160			140	240		160		
	5			40			140	200	160			100			300	124.0	62.5
	6	110	80	60		160	40	80	200	160					200		
	7	90	80	100		120	60	100		180		180	140		200		
	8	100				160		200	160	100		120	140		140		
	9	40	40			130	60		120	180		100	140				
	10	60		20		40	40		240	200		140	100				

As can be seen in this table, EFV in the pre-menstrual phase ranged from 40 to 300 mV and the average was 124.4 mV. In the menstrual phase EFV was generally higher, ranging from 120 to 540 mV, and the average was 286.8 mV. After the menstruation, EFV decreased to the same level as in the pre-menstrual phase; the values ranged from 20 to 300 mV, and the average was 124 mV. The difference between EFV obtained in

the menstrual phase and that obtained in the pre- or post- menstrual phase was shown to be statistically significant. The data illustrated in Fig. 1, in which EFV obtained from 6 subjects are plotted against the days of the

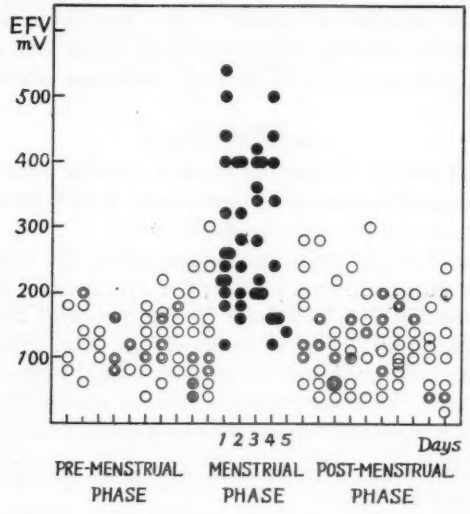

Fig. 1. Changes of EFV obtained from 6 subjects in the menstrual cycle. Ordinates: EFV in mV. Abscissae: Time in days in the menstrual cycle. Empty circles: Values obtained before and after the menstruation. Solid circles: Values obtained in the menstrual phase.

menstrual cycle. It is to be noted that the fluctuation of EFV is more remarkable during the menstrual phase than in the other phases. Despite the greater standard deviation of EFV during the menstrual phase, the increase of EFV in this phase was always so marked that the increase has proved to be statistically significant. In some cases EFV increased gradually as the menstrual phase was approached, while in the other cases an abrupt increase set in on the 1st day of menstruation. In the same way, the decrease of EFV to the normal level was found either abrupt or gradual as the case might be. In one and the same subject EFV was found different from day to day, as can be seen in Table I, but such daily variations never exceeded the variation based on the menstrual cycle.

Discussion

From the above-mentioned experiment there is no doubt that EFV increases in the menstrual phase. On the other hand it is known that an increase of EFV occurs under various unfavorable conditions such as

insufficient oxygen supply,[16] fatigue after physical and mental exercises,[20),24] etc. Therefore it may be said that the increase of EFV obtained above is an indication of a sub-normal state of the subjects in the menstrual phase. The present experiment has thus provided further evidence that the method of electric flicker represents one of the most sensitive methods for finding out sub-normal states and therefore serves as a convenient tool for survey of general conditions of subjects.

Summary

The value of electric flicker (EFV) of 6 healthy female subjects aged 16 years were measured, extending over 2 and a half months to examine the effect of the menstrual cycle on EFV.

EFV increased in the menstrual phase above the values obtained in the pre- and the post-menstrual phases; the average of EFV during menstruation was 286.8 mV, while those in the pre- and the postmenstrual phases were 124.4 and 124 mV respectively. The difference between the values for the menstrual and the non-menstrual phases was found statistically significant. This fact provides further evidence that the method of electric flicker is a sensitive method to find subnormal states of subjects.

We are greatly indebted to Prof. K. Motokawa for his many valuable discussions and kind guidance.

References

1) Sahler, T., Wien. klin. Wschr., 1924, **37**, 37.
2) Kato, A., Zbl. f. Gyn., 1933, **57**, 1804.
3) Däring, G. K. & Weber, G., Arch. f. Gyn., 1951, **179**, 442.
4) Guterman, H. S. & Talsky, A. S., Am. J. Obst. Gyn., 1949, **58**, 495.
5) Benedict, F. C. & Finn, M. D., Am. J. Physiol., 1928, **86**, 59.
6) Rubenstein, B. B., *ibid.*, 1937, **119**, 635.
7) Zuck, T., Am. J. Obst. Gyn., 1938, **36**, 998.
8) Zuck, T. & Duncan, R. L., *ibid.*, 1939, **38**, 310.
9) Galler, F. C., Münch. med. Wschr., 1940, **87**, 1110.
10) Snodgrass, J. M., Rock, J. & Menkin, M. F., Am. J. Physiol., 1943, **140**, 394.
11) Ando, K., J. Science of Labour (Jap.), 1952, **26**, 169.
12) Motoda, N., Shinjo, G. & Kiyonaga, M., *ibid.*, 1952, **26**, 169.
13) Nishikawa, Y. & Kaneko, F., *ibid.*, 1954, **28**, 381.
14) Terauchi, Y., J. Jap. Obst. Gyn. (Jap.), 1950, **2**, 87.
15) Omaru, I., Morita, M. & Horikawa, Y., J. Kurume Med. Assoc. (Jap.), 1946, **9**, 65.
16) Motokawa, K. & Iwama, K., Tohoku J. Exp. Med., 1949, **50**, 319.
17) Motokawa, K. & Suzuki, K., Jap. Med. J., 1948, **1**, 200.
18) Mita, T., Abe, Z. & Tzoe, B., Tohoku J. Exp. Med., 1951, **54**, 45.
19) Suzuki, K., *ibid.*, 1950, **52**, 9.
20) Suzuki, K., *ibid.*, 1949, **50**, 319.
21) Tsukahara, S. & Ebe, M., Kagaku (Jap.), 1949, **19**, 426.
22) Tsukahara, S., Ebe, M. & Isobe, K., Tohoku Ig. Z. (Jap.), 1949, **41**, 28.
23) Abe, Z. & Tzoe, B., *ibid.*, 1950, **46**, 49.
24) Suzuki, K., Tsukahara, S., Kobayashi, M. & Umetsu, J., *ibid.*, 1954, **49**, 313.

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Studies on Cirrhosis of the Liver

I. Production and Treatment of Fatty Liver Cirrhosis of Albino Rats

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Recently there is a growing conviction that viral hepatitis plays an important role in the causation of cirrhosis of the liver. However, all hepatic cirrhosis is not ascribed to viral hepatitis.

The development of cirrhosis from fatty liver is strongly supported by American and European investigators from the experimental studies in choline-deficient rats and clinical observations. In this country Ueda[1] reported 8 cases of fatty cirrhosis among 28 cirrhotic patients. The author experienced 2 clinical cases and 3 autopsied cases. Although Amano[2] maintains that fatty cirrhosis is rarely found in post-mortem material, it seems to be not a rare disease in Japan.

For the purpose of reexamining the problem of the production and treatment of fatty liver cirrhosis, fatty liver cirrhosis was produced in rats by feeding a choline-deficient diet, and then these rats received the treatment of high protein diet with added choline. An attempt was made to evaluate the effects of protein and choline not only by histological examination but also by determinations of liver collagen, liver fat, and serum protein.

Experimental

Methods

Fifty-two male albino rats with an average weight of 150 g. were used. They were fed with a choline-deficient diet for 8 months. Some of the rats were then killed and the remaining animals were fed with the therapeutic diet containing 30 per cent casein and 40 mg. of choline chloride and killed after 6 months. Histological examination and determinations of liver fat, liver collagen, and serum protein were all carried out on these animals. For normal controls, 14 male albino rats with an average weight of 130 g. were used. These rats were fed with a diet

similar to the therpeutic diet for 3 to 4 weeks, and were then sacrificed.

The composition of experimental diets employed is shown in Table I. Determination of liver fat was carried out by the method described

TABLE I

Composition of Diets

Constituent	Choline-deficient diet	Therapeutic diet	Diet for normal control rats
Casein	8%	30%	30%
Sucrose	48%	61%	61%
Lard	38%	–	–
Cod liver oil	2%	–	–
Soybean oil	–	5%	5%
Osborn-Mendel's Salt Mixture	4%	4%	4%
Choline chloride	–	40 mg/day	20 mg/day
Thiamin	50 μg/day	50 μg/day	50 μg/day
Riboflavin	50 μg/day	50 μg/day	50 μg/day
Pyridoxin	20 μg/day	20 μg/day	20 μg/day
Calcium pantothenate	100 μg/day	100 μg/day	100 μg/day
Nicotinamide	50 μg/day	500 μg/day	500 μg/day
Percomorph liver oil	–	6 drops/week	6 drops/week
α-tocopherol	10 mg/week	5 mg/week	5 mg/week

by Kumakawa and Sudo, and that of liver collagen was done by the method of Lowry.[3] Serum protein was estimated by the method of paper electrophoresis and refractometry. Paper-electrophoretic analyses were made using phosphate buffer of pH 7.8.

Results

Body weight, food intake, and deaths of rats

Fig. 1 illustrates the curves of average body weight and food intake as well as deaths of rats. The body weight curve of the rats on the choline-deficient diet rised gradually and attained a weight of 180 g. after 10 weeks. Then there was no fluctuation of the body weight curve for 9 weeks. From the 20th week it declined slowly up to the initial weight after 8 months. When their diet was changed from the choline-deficient diet to the therapeutic diet, there was an abrupt acceleration of growth of the rats. After 18 weeks their body weight reached 333 g., and thereafter there was no increase of body weight.

The food intake of the choline-deficient rats remained unchanged for 10 weeks, and had a tendency to decrease from the 11th week. The

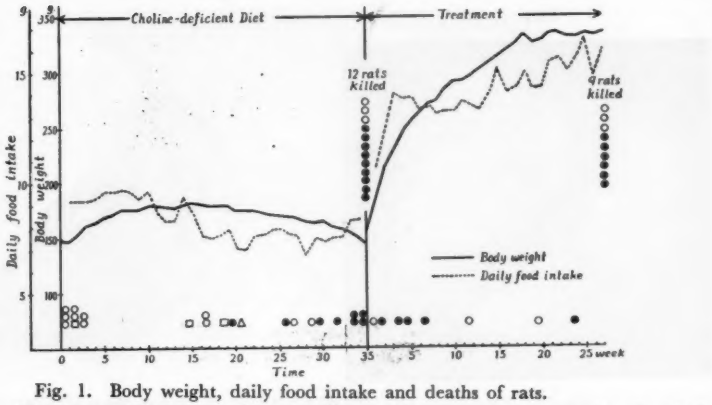


Fig. 1. Body weight, daily food intake and deaths of rats.
(Deaths: ● fibrosis, □ kidney injury, △ massive hepatic necrosis, ○ other)

average daily food intake was 8.3 g. The food intake of the rats receiving the therapeutic diet had a general trend to increase, the average intake being 14.1 g.

As is shown in Fig. 1, fibrosis of the liver was first recognized histologically in a rat which died after 18 weeks, and macroscopically in a rat which died after 25 weeks. The deaths of rats from fibrosis increased as they losed weight. Certain number of the rats on the choline-deficient diet developed alopecia from the 11th week, and after 8 months about half of them suffered from alopecia and the rats generally became ill. The change to the therapeutic diet brought prompt recovery, and alopecia disappeared in 2 to 5 weeks.

During feeding the choline-deficient diet, 10 mg. of α-tocopherol per week was given to prevent dietary massive necrosis of the liver. When α-tocopherol was decreased to 5 mg. per week for a short period, a rat developed massive necrosis and died.

Pathology

The liver of the rats fed with the choline-deficient diet was swollen and showed pale yellow color. These macroscopic findings of fatty liver were observed in almost all the rats which were fed with the choline-deficient diet for more than 4 months. The liver of about one-quarter of the rats showed finely granular surface and increased consistency, suggesting fibrosis of the liver macroscopically. (Fig. 2) In the treated rats macroscopic findings of fatty liver were not observed, but finely granular liver was still present. Marked nodular hyperplasia developed in a rat fed with the therapeutic diet for 6 months. (Fig. 3)

Histologically fatty metamorphosis was the most conspicious finding

Fig. 2

Fig. 3

Fig. 2. Fatty liver cirrhosis of a rat fed with the choline-deficient diet for 6 months. Surface is finely granular, and consistency is increased.

Fig. 3. Liver of a rat fed with the therapeutic diet for 8 months, showing nodular hyperplasia of Lobus caudalis dexter. The liver is injected with India ink through the hepatic vein.

in the choline-deficient rats. Fatty metamorphosis began in centrolobular areas and finally fatty cysts occupied almost all parenchyma. Fatty metamorphosis appeared in all the rats fed with the choline-deficient diet for more than 4 months. In the livers of the rats which died after the relatively long period of starvation, only a few number of fatty cysts were found even when they were fed with the choline-deficient diet for more than 4 months.

Besides fatty metamorphosis, "ceroid" described by Lillie[4] appeared. This material occurred in round and oval globules, and was pale yellow with hematoxylin and eosin stain and brownish-orange when stained with sudan III. It was still stained with sudan III in paraffin sections. Ceroid was scattered when it first appeared. As ceroid became marked, it formed trabeculae which divided the lobules of the liver. This ceroid seemed to be phagocytized by Kupffer's cells and was present in sinusoids. The reticular fibers of the sinusoids which were filled with ceroid had the tendency to formation of collagenous fibers.

Ceroid appeared first in centrolobular and subcapsular areas and around hepatic veins as same as fatty metamorphosis did, and later periportal areas were involved. In some rats ceroid accumulation was so marked in the areas near the hilus of the liver that the parenchyma of those areas were totally replaced by ceroid and collagenous fibers. Although fibrosis generally developed with ceroid, in some instances only collagenous fibers were present without the appearance of ceroid. Generally the proliferation of connective tissue was not marked. (Fig. 4)

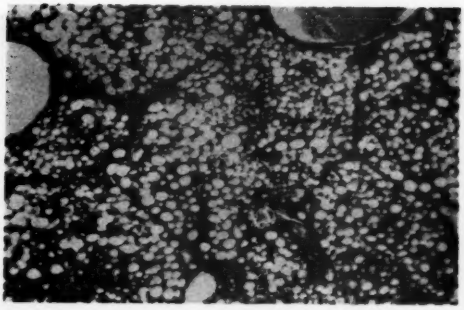


Fig. 4. Microphotograph of the liver of a rat fed with the choline-deficient diet for 8 months. Van Gieson's stain. ×64.

Fibrosis began in non-portal areas as same as ceroid accumulation. Trabeculae of collagenous fibers and ceroid linked with adjacent central veins and surrounded the periportal triads. Later the periportal triads were also involved.

As fibrosis and ceroid accumulation progressed, trabeculae of connective fibers and ceroid divided heaptic lobules and formed pseudolobules. Thus the normal architecture of liver lobules was destroyed.

On the other hand regeneration of liver cells was noticed in some rats with fibrosis. Proliferation of bile ducts developed in the rats in which fibrosis and ceroid accumulation were advanced. In some instances atypical hyperplasia of bile ducts was present. Inflammatory cells were found only slightly.

The change of the choline-deficient diet to the therpeutic diet brought the rapid disappearance of liver fat and regeneration of liver cells. In some rats fibrosis was so delicate and without the presence of ceroid that the resorption of collagenous fibers and ceroid to a certain extent were conceivable. Nevertheless in other many rats fibrosis and ceroid accumulation were still marked. (Fig. 5) Generally no statistically significant changes were noticed in fibrosis and ceroid accumulation during the treatment, although the decrease of fatty metamorphosis was obvious. (Table II)

In the liver of a rat which died from massive hepatic necrosis, diffuse coagulation necrosis of liver cells and hemorrhage were both noticed.

In the spleen ceroid was phagocytized by reticular cells around white pulps, in white pulps, and in splenic cords. Fibrosis of white and red pulps developed in a treated rat.

The renal lesion of the rats with hemorrhagic degeneration of Griffith[5] was characterized by dark and enlarged kidneys, necrosis and degeneration of tubules, hemorrhages in capsules and cortex, and cast on the one hand,

Fig. 5. Microphotograph of the liver of a rat which received treatment for 6 months. Van Gieson's stain. ×68.

TABLE II

Fatty Metamorphosis, Ceroid Accumulation and Fibrosis of the Liver Estimated Histologically

Group	No. of rats	Fatty Metamorphosis					Ceroid Accumulation					Fibrosis				
		0	+1	+2	+3	+4	0	+1	+2	+3	+4	0	+1	+2	+3	+4
Normal control rats	14	14	0	0	0	0	14	0	0	0	0	14	0	0	0	0
Rats died during the first 4 months of the choline-deficient diet	11	9	0	1	1	0	11	0	0	0	0	11	0	0	0	0
Rats diet during the second 4 months of the choline-deficient diet	12	0	4	3	3	2	2	3	3	3	1	3	2	6	1	0
Rats fed with the choline-deficient diet for 8 month	12	0	1	5	4	2	3	3	5	1	0	3	2	6	1	0
Rats died during the treatment	8	5	3	0	0	0	3	0	2	3	0	3	0	3	0	2
Rats treated for 6 months	9	8	1	0	0	0	1	3	4	1	0	3	2	4	0	0

and by fibrosis of cortex on the other hand. The latter seemed to be the result of scarring of the former changes. Besides these changes there was ceroid deposition in glomerulus and calcification of vessels, but glomerulosclerosis was not observed.

In the lung ceroid and fat deposited in the septa. Parenchymatous myositis, cellular infiltration and fibrosis were all present in the heart of some rats. Atrophy of testis was found in the rats in which fibrosis was marked.

Liver fat

Among the rats died within the first 4 months which were fed with the choline-deficient diet, only a few rats showed the increase of liver fat, probably because of their small food intake. Their average daily food intake was 4.3 g.

Liver fat increased over normal range in all the rats which died during the second 4 months and those which were killed thereafter. The average fat content of the liver of the rats which died within the second 4 months of the choline-deficient diet was 23.0 per cent, and that of the rats killed after 8 months was 24.8 per cent. Thus the increase of liver fat in the rats fed with the choline-deficient diet for more than 4 months was clearly demonstrated. (Table III)

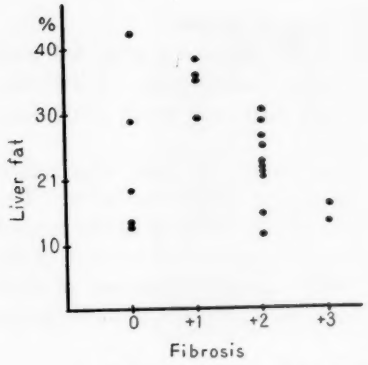
TABLE III

Liver Weight, Liver Fat and Liver Collagen

Group	No. of rats	Average final body weight, g.	Average weight of liver, g.	Liver weight as per cent of body weight	No. of rats	Liver fat as per cent of liver	No. of rats	Liver collagen as per cent of liver
Normal control rats	14	216.9	9.6	4.4	13	6.2	12	0.30
Rats died during the first 4 months of the choline-deficient diet	11	90.4	5.5	6.2	6	10.8	—	—
Rats died during the second 4 months of the choline-deficient diet	12	86.0	8.3	9.0	9	23.0	—	—
Rats fed with the choline-deficient diet for 8 months	12	137.4	9.7	7.5	12	24.8	11	0.33
Rats died during the treatment	8	160.0	9.3	6.0	7	6.4	—	—
Rats treated for 6 months	9	334.6	13.4	3.9	9	6.4	9	0.33

Dietary treatment was followed by the prompt and marked decrease of liver fat. The fat content was within normal range in all the rats which died during the treatment except two rats which died after 2 and 14 days and in all the rats which were killed after treatment except a rat which showed only mild increase of liver fat.

The relation of fat content to fibrosis or ceroid accumulation is interesting, as shown in Figs. 6 and 7. The differences of content of liver fat between the group of minimal fibrosis and that of moderate fibrosis, between the group of minimal ceroid accumulation and that of moderate ceroid accumulation, between the group of moderate ceroid accumulation and that of advanced ceroid accumulation were statistically significant. Thus the content of liver fat decreased as fibrosis or ceroid accumulation



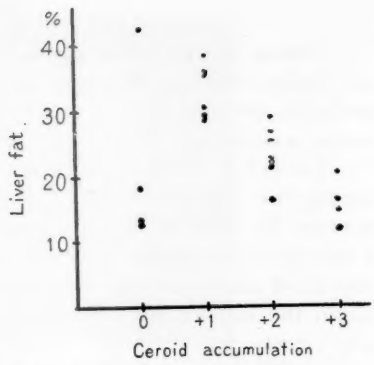


Fig. 6. The relation between fibrosis of the liver and liver fat in the rats fed with the choline-deficient diet for more than 4 months.

Fig. 7. The relation between ceroid accumulation and liver fat in the rats fed with the choline-deficient diet for more than 4 months.

progressed.

The degree of fatty metamorphosis which was estimated histologically was proportional to the content of liver fat which was determined chemically.

Collagen content

Since liver collagen content was determined in a few rats of those which died during the feeding of the choline-deficient and therapeutic diets, comparisons were made between the normal control rats, the rats fed with the choline-deficient diet for 8 months, and the rats fed with the therapeutic diet for 6 months. Their average collagen contents were 0.30, 0.33, and 0.33 per cent respectively.

When tested by Fisher's " t " test, the differences of collagen content between the normal control rats and the rats which were fed with the choline-deficient diet for 8 months, between the rats which were fed with the choline-deficient diet for 8 months and the rats treated for 6 months were all statistically significant. (Table III)

Serum protein

The differences of total protein concentration between the three groups were statistically significant. (Table IV) Total protein concentration was found decreased in the rats which were on the choline-deficient diet, and increased over normal value in those which were on the therapeutic diet.

Albumin concentration was found decreased markedly in those which

TABLE IV
Serum Protein

Group	No. of rats	g/dl					No. of rats	%				A/G
		Total	Al.	α-Gl.	β-Gl.	γ-Gl.		Al.	α-Gl.	β-Gl.	γ-Gl.	
Normal control rats	14	6.64	2.89	0.74	1.36	1.65	14	43.6	11.1	20.5	24.8	0.77
Rats fed with the choline-deficient diet for 8 months	9	6.10	2.13	0.76	1.37	1.84	21	33.0	13.4	22.5	31.1	0.50
Rats treated for 3 months	—	—	—	—	—	—	11	33.5	11.7	22.8	32.0	0.51
Rats treated for 6 months	9	7.69	2.99	0.85	1.55	2.29	9	38.9	11.1	20.1	29.9	0.64

were on the choline-deficient diet, and returned to normal in those which were on the therapeutic diet.

There were no significant changes in α-globulin concentration.

β-Globulin concentration remained unchanged in those on the choline-deficient diet. The difference of β-globulin concentration between the treated rats and the control rats was statistically significant, whereas the difference between the treated rats and the choline-deficient rats was not significant.

γ-Globulin concentration was not found increased in those which were on the choline-deficient diet, and on the contrary was increased significantly in those which were on the therapeutic diet.

After 3 months of the treatment only percentage of the serum protein was estimated. Judging from the results, the changes of serum protein of the treated rats seemed to be more pronounced in the first 3 months than in the second 3 months.

Albumin-globulin ratio was found lowered in those which were on the choline-deficient diet, and remained unchanged in the first 3 months of the treatment. In the second 3 months of the treatment albumin-globulin ratio rised, but failed to return to normal.

In the rats which were fed with the choline-deficient diet for 8 months there were no statistically significant correlations between albumin and γ-globulin concertration, between albumin concentration and fibrosis or ceroid accumulation, and between γ-globulin concentration and fibrosis or ceroid accumulation.

DISCUSSION

The histological changes in this experiment were characterized by the fibrosis which began in centrolobular areas and ceroid accumulation.

The non-portal distribution of fibrosis in experimental cirrhosis were recognized by Ashburn and his associates[6] and confirmed by other investigators.[7),8),9)] In this regard the difference of experimental cirrhosis from human cirrhosis needs to be discussed.

According to Popper and his associates,[10)] fatty cirrhosis of man is portacentral, and necrosis and inflammation in periportal and intralobular areas and membrane formation in stress fissures caused by uneven expansion of hepatic territories were more important processes of development of fibrosis than the collapse of fatty cysts. In this experiment the former two processes were not observed. Since fibrosis began in the areas of the most pronounced fatty metamorphosis and fat content decreased as fibrosis progressed, a close relation seems to be present between fibrosis and fatty metamorphosis itself. Hence the process of development of fatty cirrhosis in rats appears to be different from that of man.

However, in the experiences of the present author in human liver cirrhosis there have been no findings which indicated, as Popper and his associates described, that periportal and intralobular necrosis and inflammation and membrane formation were the main processes of development of fibrosis. Further investigation is required on the problem concerning the processes of development of fibrosis in man.

Deposition of ceroid in large quantities in livers seems to be due to the use of cod liver oil and lard in the diet.[7)] Since experimental fatty cirrhosis without ceroid can be produced on certain diets,[7),11)] it is unlikely that ceroid plays a role in the causation of fibrosis in experimental cirrhosis, and that the presence of ceroid in experimental cirrhosis is an evidence against identity with human cirrhosis.

The coexistence of both fibrosis and ceroid in most cases suggests that the same cause as that of fibrosis, such as the rupture of fatty cysts described by Hartroft,[12)] contributes to the formation of ceroid in certain conditions, for instance, when cod liver oil or lard is used.

The determination of liver fat content clearly shows the lipotropic activity of choline and high protein diet. Noteworthy is the relation of fat content to fibrosis and ceroid accumulation. Fat content decreased as fibrosis and ceroid accumulation progressed. This contributes, as well as the prompt decrease of fat after the treatment and starvation, to the rapid disappearance of the characteristics of fatty cirrhosis. Caution must be taken about this point in the investigation of etiology of liver cirrhosis.

In this experiment collagen content was expressed in per cent of wet liver weight as it was considered to be most adequate. To evaluate the changes of collagen content the influences of changes of liver fat and body weight on collagen content must be considered. Since the increase of liver fat decreases apparent collagen content, the collagen content of

the rats which were fed with the choline-deficient diet seems to be actually increased over the above-mentioned figures, and that of the treated rats seems to be actually decreased below the above-mentioned figures. However, the collagen content of the livers of the treated rats was increased over that of the normal control rats, whereas the fat content of the former was the same as that of the latter. Therefore, the changes of liver collagen similar to that of the choline-deficient rats seem to be not present in the treated rats.

According to Harkness and Harkness[13] liver collagen (mg.) in normal rats is proportional to liver weight (g.).[1.16] When liver collagen (mg.)/liver weight (g.)[1.16] is calculated, it is 2.08 in the normal control rats, 2.28 in the choline-deficient rats, and 2.20 in the treated rats. The degree of decrease in the treated rats is mild as compared with the degree of increase in the choline-deficient rats.

Thus, when the influences of liver fat and body weight on the collagen content are considered, the decrease of collagen content in the treated rats is not clearly demonstrated, whereas the increase of collagen content in the choline-deficient rats is apparent.

There have been no reports on the changes of serum protein in the experimental cirrhosis. When the results in this experiment are compared with those in clinical cases with liver cirrhosis[1),14),15),16),17),18)] or fatty liver,[10)] the former are generally accorded with the latter.

It may be based upon the fact that fibrosis is of mild degree and that, as reported by Ueda,[1)] the cirrhosis is a fatty cirrhosis, that the difference of γ-globulin concentration between the choline-deficient rats and the normal rats was not significant. The changes of serum protein on the therapeutic diet are in accord with those in clinical cases.[1),15),19),20)]

In clinical cases when they are examined by serial biopsies fibrosis are reported to remain unchanged after dietary and lipotropic treatment,[19),21),22),23),24),25),26)] whereas in experimental cirrhosis decrease of fibrosis was reported by some investigators.[9),27),28),29),30),31),32)]

In this experiment the decrease of fibrosis was not demonstrated in agreement with the results in many clinical cases and the experiment of Lowry.[33)] The decrease of fibrosis was further confirmed by the determination of collagen content of livers. On the basis of the experimental findings it is suggested that the decrease of fibrosis after the dietary and lipotropic treatment can not be expected once fibrosis has progressed to a certain extent, though the functional and morphological improvement of liver cells many occur.

Summary and Conclusions

1. Albino rats were fed with a choline-deficient diet for 8 months.

Some of the rats were then killed and the remaining animals were fed with the therapeutic diet containing 30% of casein and 40 mg. of choline chloride for 6 months. Microscopic examination and quantitative determination of liver fat, liver collagen and serum protein were carried out on these animals.

2. The liver of the rats which were fed with the choline-deficient diet was finely granular, and microscopic examination showed fatty metamorphosis, ceroid accumulation, and fibrosis of the liver which began in non-portal areas. The dietary treatment was followed by the rapid disappearance of fat, but there were no recognizable effects on fibrosis and ceroid accumulation.

3. Liver fat increased remarkably on the choline-deficient diet, and returned to nearly normal level on the therapeutic diet. In the rats which were fed with the choline-deficient diet for more than 4 months liver fat decreased as fibrosis or ceroid accumulation progressed.

4. Liver collagen increased on the choline-deficient diet, but the decrease on the therapeutic diet was not clearly demonstrated.

5. On the choline-deficient diet serum albumin decreased markedly, but the increase of γ-globulin was not statistically significant. After the dietary treatment albumin returned to normal and γ-globulin increased significantly. Albumin-globulin ratio was lowered on the choline-deficient diet. On the therapeutic diet albumin-globulin ratio rised significantly, but failed to return to normal.

References

1) Ueda, Nihon Naika-gakkai Zasshi (Jap.), 1953, **42**, 591.
2) Amano, Yamamoto & Dohi, Sogo Rinsho (Jap.), 1952, **9**, 230.
3) Lowry, Gilligan & Katersky, J. Biol. Chem., 1941, **139**, 795.
4) Lillie, Ashburn & Lowry, Publ. Health Rep., U.S.P.H.S., 1942, **57**, 502.
5) Griffith & Wade, J. Biol. Chem., 1939, **131**, 567.
6) Ashburn, Endicott, Daft & Lillie, Am. J. Path., 1947, **23**, 159.
7) György & Goldblatt, J. Exp. Med., 1949, **89**, 245.
8) Hartroft, Anat. Rec., 1950, **106**, 61.
9) Plough, Patek & Bevans, J. Exp. Med., 1952, **96**, 221.
10) Popper, Szanto & Elias, Gastroenterology, 1955, **28**, 183.
11) Endicott, Daft & Sebrell, Proc. Soc. Exp. Biol. Med., 1944, **57**, 330.
12) Hartroft & Ridout, Am. J. Path., 1951, **27**, 951.
13) Harkness & Harkness, J. Physiol., 1954, **125**, 447.
14) Popper, Bean, de la Huerga, Franklin, Tsumagari, Routh & Steigmann, Gastroenterology, 1951, **17**, 138.
15) Post & Patek, Arch. Int. Med., 1942, **69**, 67.
16) Rafky, Weingarten, Krieger, Stern & Newman, Gastroenterology, 1950, **14**, 29.
17) Ricketts, Sterling, Kirsner & Palmer, *ibid.*, 1949, **13**, 205.
18) Whitman, Rossmiller & Lewis, J. Lab. Clin. Med., 1950, **35**, 167.
19) Buck, *ibid.*, 1948, **33**, 555.

20) Sterling, Ricketts, Kirsner & Palmer, J. Clin. Invest., 1949, **28**, 1236.
21) Beams & Endicott, Gastroenterology, 1947, **9**, 718.
22) Eckhardt, Zamcheck, Sidman, Gabuzda & Davidson, J. Clin. Invest., 1950, **29**, 227.
23) Franklin, Salk, Steigmann & Popper, Am. J. Clin. Path., 1948, **18**, 273.
24) Post, Benton, Breakstone & Hoffman, Gastroenterology, 1952, **20**, 403.
25) Steigmann, J.A.M.A., 1948, **137**, 239.
26) Volwiler, Jones & Mallory, Gastroenterology, 1948, **11**, 164.
27) Cameron & Karunaratne, J. Path. Bact., 1936, **42**, 1.
28) György & Goldblatt, J. Exp. Med., 1949, **90**, 73.
29) Morrione, *ibid.*, 1947, **85**, 217.
30) *Idem*, Am. J. Path., 1949, **25**, 273.
31) Sellers, Lucas & Best, Brit. Med. J., 1948, **1**, 1061.
32) Steinberg & Martin, Arch. Path., 1946, **41**, 1.
33) Lowry, Ashburn & Sebrell, Quart. J. Stud. on Alcohol, 1945, **6**, 271.

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Studies on Cirrhosis of the Liver

II. Influences of Temperature and Nutrition on the Production of Cirrhosis of the Liver

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In previous communications[1)-8)] the production and treatment of fatty liver cirrhosis in choline-deficient rats were reported. The statistical studies on the relation between the mortality from liver cirrhosis and temperature and the experiments, in which the influence of temperature on the liver fat content of choline-deficient rats and the influence of nutrition on the production of liver cirrhosis due to carbon tetrachloride in rats were investigated in relation to the statistical studies, are described in the present paper.

The Relation Between the Mortality from Cirrhosis of the Liver and Temperature

In the statistical studies of the mortality from liver cirrhosis the relation between the mortality from liver cirrhosis and temperature attracted the attention of the authors.

Figs. 1 and 2 illustrate the age-corrected mortalities from liver cirrhosis in males and females in the prefectures of Japan. The mortalities increase, except in the Hokkaido District, from the northeast to the southwest. The relation between the age-corrected mortalities from liver cirrhosis and mean annual temperatures in the prefectures of Japan is shown in Figs. 3 and 4. The age-corrected mortalities in the prefectures of Japan are from the Department of Public Health, Tohoku University, and for the period 1947–1950. The mean annual temperatures are from Rika Nempyo,[4)] and for the period 1921–1950. The correlation coefficients are +0.502 in males and +0.456 in females. They are both

Fig. 1

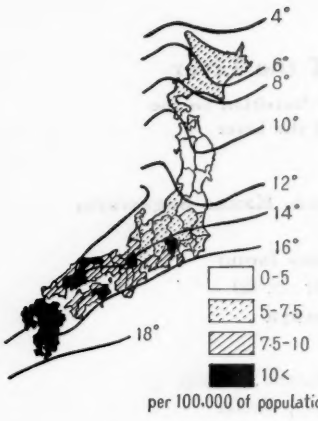

Fig. 2

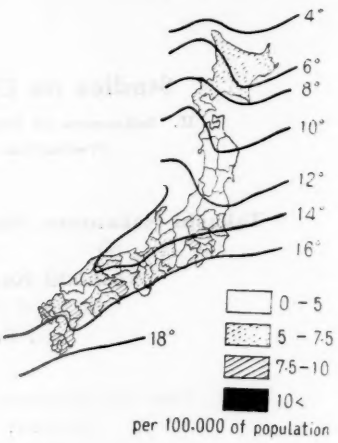

Fig. 1. The mortalities from cirrhosis of the liver in males in the prefectures of Japan. (1947–1950)

Fig. 2. The mortalities from cirrhosis of the liver in females in the prefectures of Japan. (1947–1950)

Fig. 3

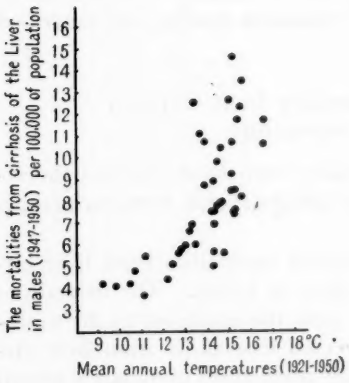

Fig. 4

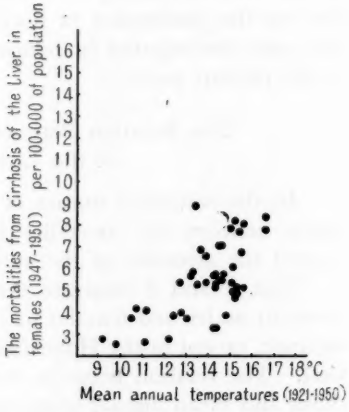

Fig. 3. The relation between the mortalities from cirrhosis of the liver in males and the mean annual temperatures in the prefectures of Japan. (Hokkaido is omitted.)

Fig. 4. The relation between the mortalities from cirrhosis of the liver in females and the mean annual temperatures in the prefectures of Japan. (Hokkaido is omitted.)

statistically significant. ($P<0.001$ and $P<0.01$) Thus the statistical observations in the prefectures of Japan indicate that the higher the temperatures the larger the mortalities from liver cirrhosis.

It is regrettable that vital statistics data are not available for the countries other than Japan except for U.S.A. The statistics for U.S.A. reveals that there is no significant correlation between the mortalities from liver cirrhosis and temperatures in the states of U.S.A.

The relation between the mortalities from liver cirrhosis[5] and temperatures[4] in the countries of the world of mean annual temperature below 16°C (Canada, U.S.S.R., Norway, Finland, Sweden, Denmark, Scotland, West German Republic, England and Wales, Holland, Ireland, France, Ugoslavia, New Zealand, Chile, Japan, U.S.A., Italy, and Portugal) are shown in Fig. 5. The correlation coefficient is +0.682, and statistically significant. ($P<0.01$)

In the countries of mean annual temperature above 17°C (Israel, Egypt, the Union of South Africa, Guatemala, Australia, Hawaii, Port Rico, Singapore, Jamaica, and Ceylon) there are no significant correlations between the mortalities from liver cirrhosis[6] and mean annual temperatures,[4] and the mortalities in these countries are generally lower than in the countries of mean annual temperature of 11° to 16°C. (Fig. 6)

Fig. 5

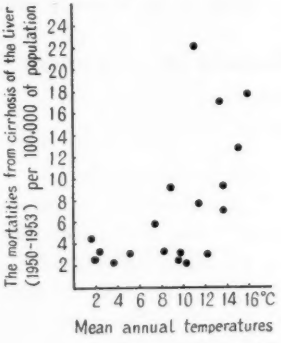

Fig. 6

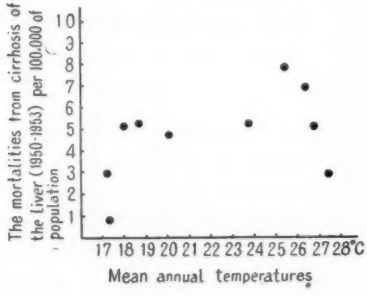

Fig. 5. The relation between the mortalities from cirrhosis of the liver and the mean annual temperatures in the countries of temperature below 16°C.

Fig. 6. The relation between the mortalities from cirrhosis of the liver and the mean annual temperatures in the countries of temperature above 17°C.

The significant correlation between the mortalities from liver cirrhosis and temperatures in the prefectures of Japan and in the countries of the world of mean annual temperature below 16°C suggest that the mortality

from liver cirrhosis is influenced by temperature by a certain mechanism.

It seems to be due to the more well-equiped heating arrangements in the Hokkaido District than in the North-eastern District that the mortality is higher in the Hokkaido District than in the North-eastern District while the temperature in the former is lower than in the latter. The low incidence of hypertension in the Hokkaido District[6] and the results of the investigation of protein intake, which will be discussed afterwards, support this hypothesis.

It also may be attributed to the well-equiped heating arrangements in American houses that significant correlation is not found between the mortalities from liver cirrhosis and temperatures in the states of U.S.A. The inadequate heating arrangements in Japanese houses seem to contribute to making the influence of temperature on the inhabitants conspicious and the correlation between the mortalities from liver cirrhosis and temperatures clear.

It appears to indicate the reduction of mortalities at higher temperatures that the mortalities in the countries of temperature above 17°C is generally lower than those in the countries of temperature of 11 to 16°C. However, further research is required about the mortalities from liver cirrhosis in the countries of temperature above 17°C, since, judging from the statistical data of autopsied materials, the mortalities in some of these countries, such as Ceylon and other countries, seem to be actually more increased than those illustrated in Fig. 6.

If the mortality from liver cirrhosis is influenced by temperature, it

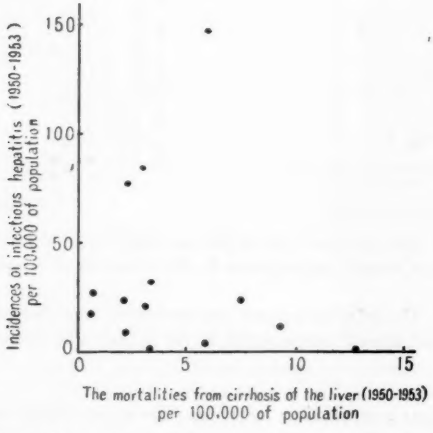

Fig. 7. The relation between the mortalities from cirrhosis of the liver and the incidences of infectious hepatitis in the countries of the world.

is of interest to know the mechanism by which temperature influences the mortality from liver cirrhosis. At first, when the mortality from liver cirrhosis is compared with the incidence of infectious hepatitis,[7] which is one of the important causes of liver cirrhosis, there is no significant correlation between the mortalities from liver cirrhosis and incidences of infectious hepatitis in the countries of the world, and no significant positive correlation between incidences of infectious hepatitis and mean annual temperatures. (Figs. 7 and 8)

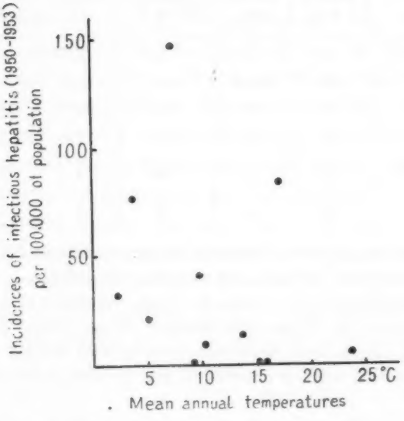

Fig. 8. The relation between the incidences of infectious hepatitis and the mean annual temperatures in the countries of the world.

Secondly, when fatty liver, which is as important cause of liver cirrhosis as viral hepatitis, is considered, there is a possibility of influence of temperature on fatty liver cirrhosis, since pseudolipotropic action of cold in the choline-deficient rats is reported by Sellers and his associates in 1952.[8] The following experiments were undertaken, therefore, with the hope of shedding further light on the influence of temperature on the liver fat content of choline-deficient rats.

The Influence of Temperature on the Liver Fat Content of Choline-deficient Rats

Experiment 1. Sixty male albino rats with an average weight of 130 g. were fed with choline-deficient diets (low and high fat diet) and kept at temperatures of 22° and 7°C for 17 to 19 days and then killed. The composition of experimental diets employed is shown in Table I. Liver fat was estimated by the method of Kumakawa and Sudo.

The results are shown in Table II. When comparisons were made between groups 1 and 2, between groups 4 and 5, which all were fed with 6% casein diet, the changes of body weight of the rats which were kept

TABLE I

The Composition of Experimental Diets

Figures express percentages

Constituent	Exp. 1*				Exp. 2†	Exp. 3*	
	Group I & II	Group III	Group IV & V	Group VI	Group I–IV	Group‡ I–IV	Group V
Casein	6	4.5	6	8.5	5	5	30
Sucrose	84	85.5	49	46.5	60.5	85	60
Lard	5	5	40	40	25	5	5
Cod liver oil	1	1	1	1	2	1	1
Osborne-Mendel's salt mixture	4	4	4	4	7	4	4
Cystine	0	0	0	0	0.5	0	0

* Daily vitamin supplements: thiamin 50 μg., riboflavin 50 μg., pyridoxine 20 μg., Ca-pantothenate 100 μg., and nicotinamide 500 μg.

† Daily vitamin supplements: thiamin 25 μg., riboflavin 12.5 μg., pyridoxine 10 μg., Ca-pantothenate 50 μg., nicotinamide 50 μg., biotin 1.5 μg., menadione 5 μg., folic acid 2.5 μg., and para-aminobenzoic acid 500 μg.

‡ When employed: choline chloride 40 mg. per day, α-tocopherol 10 mg. per week.

TABLE II

The Food Intake and Liver Fat of the Rats Fed with the Choline-deficient Diet for 17–19 Days

Group No.	Diet		Temperature (°C)	No. of rats	Change of body weight g.	Average food intake g.	Average protein intake g.	Liver fat wet %
	Protein %	Fat %						
1	6	5	22	10	+ 2.6	9.7	0.58	10.09
2	6	5	7	9	− 1.4	13.0	0.78	8.43
3	4.5	5	7	6	− 2.0	14.0	0.63	9.80
4	6	40	22	9	−12.0	8.8	0.53	10.99
5	6	40	7	7	−13.1	10.2	0.61	8.66
6	8.5	40	7	7	+ 6.0	12.0	1.02	11.34

at 7° and 22°C were nearly the same, the final body weight of the former being slightly smaller than that of the latter. Average daily food intake of the rats which were kept at 7°C was larger than that of the rats which were kept at 22°C.

On the low fat diet the liver fat content of the rats which were kept at 22°C was 10.09%, and that of the rats which were kept at 7°C was 8.43%. The difference was not statistically significant. On the high fat diet the liver fat content of the rats which were kept at 22°C was 10.99 %, and that of the rats which were kept at 7°C was 8.66%. The difference was statistically significant.

Pseudolipotropic effect of cold is, according to Sellers and his associates,[8] not likely to be through thyroid or adrenal gland. For the cause of lipotropic effect of cold the increase of daily food intake at lower temperature is considered in this experiment. At lower temperature daily food intake increases owing to the increase of metabolism. Hence the protein intake also increases. Since the metabolism, which increases to maintain the body temperature constant at lower temperature, does not require protein, the protein intake which increases at lower temperature appears to be consumed for a lipotropic substance.

Groups 3 and 6 were prepared for the purpose of investigating the relation between protein intake and liver fat content. Since the change of body weight of group 6 was considerably larger than those of groups 4 and 5, it was not possible to compare group 6 with groups 4 and 5. The comparison of group 3 with group 2 or 1 was possible because of little differences of the changes in body weight between these three groups. The protein intake of group 3, in which the percentage of the casein in the diet was reduced from 6% to 4.5%, decreased to approximately the same level as group 1. Simultaneously the liver fat content of group 3 increased to approximately the same level as group 1. Thus the prevention of deposition of liver fat was not observed in the rats which were kept at 7°C, when their protein intake decreased to the same level as that of the rats which were kept at 22°C. Accordingly it seemed to be due to the increased protein intake that the deposition of liver fat in the choline-deficient rats was prevented at lower temperature.

From the results of the present experiment in rats it is suggested that at lower temperature deposition of liver fat is prevented by the increased protein intake, which occurs as a result of the increased metabolism. Is this inference applicable to the relation between the motality from liver cirrhosis and temperature? Hence the relation between the mortality from liver cirrhosis and protein intake is investigated. Since the data concerning the protein and calorie intake of the general population in the prefectures of Japan are not available, the results of the investigation of the nutrition of peasants in agricultural districts[9] are used instead. Significant negative correlation is present between the mean annual temperatures and the protein or calorie intakes of peasants in the agricultural

districts. The correlation coefficients between the protein intakes of the peasants and the mortalities of the male and female population in the agricultural districts are −0.475 in males and −0.369 in females. They are not statistically significant. Nevertheless, when the Northern Kyushu District, in which the protein or calorie intake of peasants is too large considering its higher temperature, is omitted, the correlation coefficients are −0.794 in males, and −0.678 in females, and they are both statistically significant. (Fig. 9)

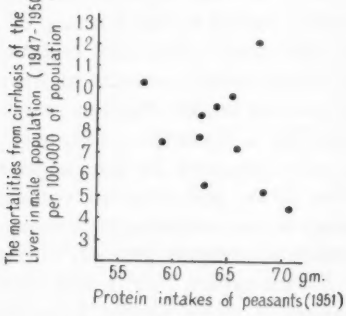

Fig. 9. The protein intakes of the peasants in agricultural districts o. Japan compared with the mortalities from cirrhosis of the liver in the male population in the same districts.

Since the data of protein intake are those of the peasants, and not those of the general population in the agricultural districts, it is not possible to draw a conclusion from the above-mentioned results. However it seems to be not very unreasonable to apply the inference which is drawn from the experiment in rats to human being in this country.

The relation between the mortalities from liver cirrhosis and mean annual temperatures is more complicated in the countries, where the composition of diets, requirements of calories, and other factors vary.

It is of interest to know whether the mortality from liver cirrhosis can be explained by the intake of protein, lipotropic substances, fat, and alcohol, and whether the mortality from liver cirrhosis is influenced by temperature through these dietary factors. It is regrettable that sufficient data are not available at present for the authors to discuss these problems. When sufficient data are available to the authors in future, the report which treats these problems will be presented.

Experiment 2. In experiment 1 the rats were kept at only two kinds of temperatures and fatty liver was mild owing to the absence of cystine in the diets. In experiment 2 one hundred and four male mice with an

average weight of 18 g. were fed with a choline-deficient diet containing 0.5% of cystine and 25% of lard and kept at four kinds of temperatures for 19 days. The temperatures at which mice were kept were 5.5°C (group 1), 12.7°C (group 2), 19.4°C (group 3), and 26.4°C (group 4).

The results of experiment 2 are shown in Table III. Decrease of body weight was observed in all the groups, and was marked especially

TABLE III

The Food Intake and Liver Fat of the Mice Fed with the Choline-deficient Diet with Cystine for 19 Days

Group No.	Temperature (°C)	No. of mice	Change of body weight g.	Average food intake g.	Liver fat wet %
1	5.5	6	−0.81	3.19	21.13
2	12.7	10	−2.36	3.19	25.07
3	19.4	17	−0.81	2.22	18.01
4	26.2	18	−5.48	1.82	24.15
Normal control mice (30% casein diet)		25	+0.87	3.01	6.49

in groups 2 and 4. The largest food intake was found in groups 1 and 2, and food intake increased as environmental temperature became higher. Liver fat increased markedly in all the groups. Although the differences of liver fat between groups 2 and 3, and between groups 3 and 4 were statistically significant, significant differences were not observed between groups 1, 2, and 4. Thus there was no regular relationship between the environmental temperature and liver fat content.

It seemed to be due to the strong hypolipotropic diet containing 0.5% of cystine that the decrease of liver fat was not observed at lower temperature whereas the intake of food and protein increased as same as in experiment 1.

The reason why the liver fat content of group 3 was lower than those of the other 3 groups remained obscure. This may be related to the fact that the mortality of group 3 was 15% while the mortalities of the other 3 groups were larger than 60%.

The Influence of Nutrition on the Production of Liver Cirrhosis Due to Carbon Tetrachloride

Since the increase of protein intake at low temperature appears to prevent the production of fatty cirrhosis, there seems to be the possibility that the increase of protein intake at low temperature may influence the production of liver cirrhosis due to carbon tetrachloride or hepatitis virus,

which injuries the liver. For the purpose of exploring this possibility and fundamental study of treatment and prevention of liver cirrhosis in general, the influences of high protein diet and other factors on the production of liver cirrhosis due to carbon tetrachloride in rats were investigated.

Sixty male albino rats with an average weight of 150 g. were divided into four groups: group 1 was fed with a low protein diet, group 2 with a low protein diet plus choline chloride, group 3 with a low protein diet plus α-tocopherol, group 4 with a low protein diet plus choline chloride and α-tocopherol, and group 5 with a high protein diet. During the feeding of the experimental diets, 13% carbon tetrachloride in olive oil was administered to all the rats by stomach tube in doses of 0.2 cc/100 g. body weight 3 times a week. After 8 weeks of the feeding of the experimental diets and the administration of carbon tetrachloride, the rats were sacrificed. Determination of liver fat was carried out by the method described by Kumakawa and Sudo, and that of liver collagen was done by the method of Lowry.[10] Serum protein was estimated by the method of paper electrophoresis and refractometry. Paper-electrophoretic analyses were made using veronal buffer of pH 8.6 and amido black 10 B.

The results are shown in Tables IV and V. The differences of the mortalities between groups 2 and 3 and between group 4 and group 2 or 3 were statistically significant. Thus the mortalities were lowered by the supplement of α-tocopherol, and elevated by the high protein diet.

TABLE IV

The Death Rate and the Incidence of Fibrosis of the Liver in Rats Given Carbon Tetrachloride for 8 Weeks

Group No.	Diet	Average initial body weight g.	Average final body weight g.	No. of the rats died (Death rate)	No. of rats which underwent liver fibrosis of grade:			
					+3	+2	+1	0
1	5% casein	156.9	148.1	7 (47%)	0	5	3	0
2	5% casein +choline	140.8	132.7	9 (60%)	0	3	2	1
3	5% casein +α-tocopherol	157.1	152.3	5 (33%)	2	0	7	1
4	5% casein +choline +α-tocopherol	147.5	147.6	3 (20%)	0	2	6	4
5	30% casein	132.5	185.8	11 (73%)	7*	2*	0	0

* Rats which died in the 7th and 8th weeks are also included.

The liver fat content of all the five groups increased over that of normal control rats. The largest increase of liver fat content was ob-

TABLE V

Liver Fat, Liver Collagen and Serum Protein of the Rats Given Carbon Tetrachloride for 8 Weeks

Group No.	Diet	Liver fat wet %	Liver collagen wet %	Serum protein g/dl				
				Al.	α_1-Gl.	α_2-Gl.	β-Gl.	γ-Gl.
1	5% casein	20.75	0.424	2.64	0.57	0.65	1.94	1.81
2	5% casein +choline	12.59	0.441	2.60	0.61	0.88	2.17	1.19
3	5% casein +α-tocopherol	19.32	0.432	2.37	0.55	0.84	1.90	1.36
4	5% casein +choline +α-tocopherol	12.84	0.427	2.43	0.57	0.67	1.81	1.20
5	30% casein	8.23	0.398	2.72	0.58	0.72	2.59	1.51
Normal control rats (30% casein diet, not given CCl_4)		6.21	0.302	3.35	0.57	0.70	1.81	1.72

served in groups 1 and 3. In groups 2 and 4, which both were supplemented with choline, the increase of liver fat was milder. The rats receiving the high protein diet showed the least increase. These results showed the lipotropic activity of the high protein diet and choline clearly.

Liver collagen content of all the five groups increased over normal value. The differences of collagen content between the five groups were not statistically significant.

Determination of serum protein indicated the significant increase of albumin concentration over normal value in all the groups except group 1, of which number of rats was small. The differences of albumin concentration between the five groups were not significant. There were no significant differences of α_1-, α_2-, β-, and γ-globulin concentration between the five groups and normal control rats, and between the five groups each other.

The comparison of fibrosis which was estimated histologically showed an interesting result. Since the number of the rats which survived over 8 weeks was only 4, calculation was made including the rats which died in more than 6 weeks. Advanced fibrosis was observed in 77.8% of group 5, and not observed in group 1. The difference was statistically significant. ($P<0.02$) Moderate fibrosis was present in 16.7% of group 4, 20.0% of group 3, and 62.5% of group 1. When 16.7% was compared with 62.5%, the possibility, at which it occurred by chance, was 5.2%. When 20.0% was compared with 62.5%, the possibility was 8.9%. Thus it was demonstrated that fibrosis of the liver of the rats which were fed with the high protein diet was more advanced than that of the rats which were fed with

the low protein diet. The supplement of α-tocopherol seemed, especially in the presence of choline, to prevent the production of fibrosis, though the tendency was not unquestionally demonstrated. Supplement of choline had no effect on the production of fibrosis.

It was in accordance with the results of the experiment in the choline-deficient rats which were previously reported by the authors[1),2),3)] that increase of liver collagen and decrease of albumin concentration were observed in the rats which were given carbon tetrachloride. When both were compared with each other, the increase of liver collagen content, which was expressed in per cent of wet liver weight, was more marked in the rats which were administered with carbon tetrachloride than in the choline-deficient rats.

Since α-tocopherol is reported to be protective against single administration of carbon tetrachloride[11)] and choline not protective against chloroform poisoning,[12)] it is easily understandable that the supplement of α-tocopherol seemed to prevent the production of fibrosis and choline had no effect on the production of fibrosis.

Hitherto there has been some disagreement, not in accordance to chloroform poisoning, on the efficacy of protein in preventing liver damage due to carbon tetrachloride. It was reported by Hove[11)] that 20% casein diet gave a greater protection against single administration of carbon tetrachloride than 10% casein diet. On the other hand Drill and his associates[13)] found greater degree of liver damage in the dogs which were fed with 20% casein diet than with 8% casein diet. Bollman[14),15)] reported a similar protective effect of low protein diet. But the composition of the diets which were employed in his experiment were not adequate to investigate the influence of protein, because meat occupied one-half, three-fourth, or total of high protein diets. In these three experiments fibrosis of the liver was not produced by the administration of carbon tetrachloride owing to the short period of observation.

In the experiment of chronic poisoning reported by György and his associates,[16)] the rats which were fed with 8% casein diet showed higher mortality than with 18% casein diet. The higher mortality of the rats on low casein diet appeared to be based more on pathologic changes in the kidney than in the liver. The effect of protein on fibrosis was not observed.

The results of the present experiment, in which 5% casein diet and 30% casein diet were used, showed the increase of mortality and progression of fibrosis in the rats which were fed with the high protein diet. Although these results are not coincident with those of György and his associates,[16)] it must be considered that the conditions were not identical.

The results of the present experiment is easily understandable, if low protein diet give, as Drill reports, greater protection against carbon

tetrachloride than high protein diet. Nevertheless, in the experiment of the present authors, in which the protective effect of 30% casein diet against the single administration of carbon tetrachloride was compared with that of 18% yeast diet, 30% casein diet was more protective than 18% yeast diet.[17] This experiment indicates the protective action of high protein diet, though yeast diet is not identical with casein diet. Furthermore Hove reports, as mentioned above, greater protection of high casein diet than that of low casein diet. Since this problem is very important with respect to the treatment and prevention of liver cirrhosis, further investigation is required.

Summary and Conclusions

1. Statistically significant positive correlation was recognized between the mortalities from liver cirrhosis and the mean annual temperatures in the prefectures of Japan. In the countries of the world significant correlation was present only in the countries of mean annual temperature below 16°C.

2. Rats were fed with a choline-deficient diet without cystine at temperatures of 7 and 22°C. On a high fat diet the liver fat content of the rats which were kept at 7°C was significantly less than that of the rats which were kept at 22°C.

At lower temperature food and protein intake increased. The prevention of deposition of liver fat was not observed in the rats which were kept at 7°C, when their protein intake was decreased to the same level as that of the rats which were kept at 22°C. Hence the prevention of deposition of liver fat at lower temperature seemed to be due to the increased protein intake.

3. The liver fat content of the mice which were fed with a choline-deficient diet with added cystine at temperature of 6°, 13°, 19°, and 26°C increased markedly. No regular relationship between liver fat content and environmental temperature was observed in these animals.

4. The influence of nutrition on the production of liver cirrhosis due to carbon tetrachloride was investigated. With the administration of carbon tetrachloride liver collagen content increased significantly and serum albumin concentration decreased significantly. Protein, choline, and α-tocopherol had no influences on the changes of liver collagen content and serum protein. On a low protein diet fibrosis of the liver which was estimated histologically was reduced by the supplement of α-tocopherol, but not influenced by the addition of choline. Fibrosis of the liver of the rats which were fed with a high protein diet was more advanced than that of the rats which were fed with the low protein diet.

References

1) Nakamura, S., Nihon Naika-gakkai Zasshi (Jap.), 1956, **45**, 250.
2) *Idem*, Tohoku J. Exp. Med., In press.
3) Nakamura, T., Nakamura, S., Sugawara, Katakura & Takizawa, Nihon Naika-gakkai Zasshi (Jap.), 1955, **44**, 398.
4) Tokyo Astronomical Observatory, Rika Nempyo (Jap.), 1956, **29**, Meterolog. pp. 7 and 78.
5) United Nations, Demographic Yearbook, 1954, **6**, 558.
6) Takahashi, Sasaki, Takeda & Ito, Nihon Iji Shimpo (Jap.), 1955, No. 1629, 27.
7) World Health Organization, Epidemiological & Vital Statistics Report, 1954, **7**, 97.
8) Sellers & You, Bioch. J., 1952, **51**, 573.
9) The Statistics & Survey Division of Japan Ministry of Agriculture & Forestry, Report on the nutrition of peasants for the year 1951 (Jap.), 1953, p. 11.
10) Lowry, Gilligan & Katersky, J. Biol. Chem., 1941, **139**, 795.
11) Hove, Arch. Bioch., 1947, **17**, 467.
12) Miller & Whipple, J. Exp. Med., 1942, **76**, 421.
13) Drill, Loomis & Belford, J. Indust. Hyg. Toxicol., 1947, **29**, 180.
14) Bollman, Ann. Int. Med., 1938, **12**, 1.
15) *Idem*, J.A.M.A., 1943, **121**, 1413.
16) György, Seifter, Tomarelli & Goldblatt, J. Exp. Med., 1946, **83**, 449.
17) Nakamura, S., Sugawara, Katakura & Isono, To be published.

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Nucleic Acids

VIII. Chemical Comparison between DNAs of Human Placenta and of Human Spleen

First Report: On Composition and Physical Properties

By

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In a foregoing article,[1] Masamune *et al.* concluded that the DNA of Yoshida sarcoma is serologically "immature." Since a large amount of cancer DNA, which is difficultly accessible, is requisite for chemical study of its structural characteristics, it was replaced with the DNA of placenta, which grows rapidly and whose components show more intensive skin reaction in cancer patients than in the non-cancerous[2)-6)] like as those of cancer tissue[7),8)]. It was compared with DNA of human spleen, because this appears to be species-specific but perfectly devoid of organ-specificity.[1]

Chargaff[9),10)] claimed species-specificity of DNAs from their bases contents, although the assertion is not strictly applicable as already mentioned by Masamune and others.[1] As to Cancer DNA, Chargaff & Lipshitz[10)] found no divergency of purine and pyrimidine contents of DNA in a cancer metastatic portion of liver of a patient from the corresponding contents of those in the unaffected portion of the cancered liver and of liver of a non-cancerous patient. Woodhouse[11)] made a similar study on DNAs of cancers in rats and mouse (spontaneous, transplanted and chemically induced) and of human uterus and chest cancers and said that some of those DNAs contained bases in amounts different from those in DNAs from normal organs although both the normal and tumor DNAs belonged to the mammalian composition type.

Experiments and Comments

Isolation of the DNAs as Sodium Salts

Human Splenic DNA

Prep. 1. A human spleen weighing 203 g. was washed with ice-chilled physiological saline as soon as possible after autopsy, cut into small

pieces, and after freezing in an ice-salt mixture, crushed. It was then homogenized with 3 volumes of 0.14 *M* NaCl[12] containing 0.01 *M* sodium citrate, and the centrifugate was further treated 6 times in a similar manner. The white insoluble residue was suspended in water, and after making up to 600 cc. with water, added to with solid NaCl to 10%. The mixture was shaken for 18 hours at below 5°C and centrifuged at 2,200×g for 30 minutes. The supernatant was added to with additional NaCl to 2.6 molarity and poured into 2 volumes of 95% alcohol. The non-colored fibers of crude DNA that precipitated was squeezed, dissolved in 10% NaCl by means of a homogenizer and deproteinized by 8 repetitions of Sevag procedure. Then the viscid solution was precipitated with an equal volume of 99% alcohol, washed with 66% alcohol thoroughly to remove NaCl at first and afterwards with 95, 99 and 100% alcohol in turn and desiccated in vacuo over anhydrous calcium chloride. Yield 750 mg.

Preps. 2 and 3. In a manner similar to above, Prep. 2 was obtained from an 80 g. portion of a spleen and Prep. 3 from a 60 g. portion of another spleen. The yields were 342 and 253 mg. respectively.

Prep. 4. 130 g. in total of mixed portions of two human spleens were washed with chilled physiological saline, freezed and homogenized with 10% NaCl (350 cc.) as above. The centrifuged supernatant was poured into 10.4 volumes of ice-chilled water. The white fibrous precipitate (DNP) was dissolved in 1 *M* NaCl containing 0.01 *M* sodium citrate and poured into 6 volumes of ice-chilled water. The fibers given here were submitted to the same treatment twice more and further to Sevag procedure and other operations described above. Yield 350 mg.

Prep. 5. It was prepared from a 70 g. portion of a human spleen like as Prep. 4 with a few modifications :—10% NaCl was substituted with 1 *M* NaCl containing 0.01 *M* sodium citrate to extract DNP and the solution was poured into 6 volumes of ice-chilled water. Dissolution and precipitation was repeated twice more, and the fibers given were precipitated from 10% NaCl by pouring into 2 volumes of alcohol, followed by deproteinization according to Sevag (repeated 8 times) and subsequent operations above. Yield 250 mg.

Human Placental DNA

Preparation of the placental DNA was carried out at below 5°C throughout the whole course in winter, because, otherwise, it decomposes easily during isolation probably due to the presence of an abundant quantity of DNAase in this organ.

Prep. 1'. A fresh placenta was washed under running water and with physiological saline in turn until no more blood was removed, freed

from the adhering membrane and blood vessels, and after freezing, crushed. The pieces weighing 500 g. were homogenized with 3 volumes of a 0.14 *M* NaCl containing 0.01 *M* sodium citrate and centrifuged. The supernatant was discarded. The centrifugate was suspended in 4 volumes of the same salt solution, strained through a wire gauze of 1.7×1.7 mm. mesh to eliminate coarse pieces and centrifuged again. Then it was washed with the salt solution again and again in a similar manner without percolation. The almost decolorized residue was added to with water up to 500 cc. and with solid NaCl to 1.2 molarity, and shaken for 18 hours, followed by centrifugation ($2{,}200 \times g$; 30 min.). The supernatant was added to with additional NaCl to 2.6 molarity and poured into 2 volumes of 95% alcohol. The crude DNA given was freed from protein, washed and dried as described in the case of Prep. 1 of spleen DNA. Yield 227 mg.

Preps. 2′ and 3′. Each of them was prepared starting with two placentas. Yielded were 337 and 313 mg. from 670 and 650 g. of freezed tissues respectively.

Prep. 4′. The preparation procedure was generally the same as the one above, but to the solution given by extraction of the tissue with 1.2 *M* NaCl was added additional solid NaCl to 10% before pouring into 2 volumes of 95% alcohol. 130 mg. of the DNA were yielded from one placenta (freezed tissue 400 g.).

Prep. 5′. 460 g. of freezed tissue were obtained from two placentas in the manner above. It was homogenized with 3 volumes of a 0.14 *M* NaCl containing 0.01 *M* sodium citrate and centrifuged ($1{,}600 \times g$, 30 min.). The centrifugate was suspended in 1 *l.* of the same salt solution and after passing through the wire gauze, centrifuged. The centrifugate was washed twice more by homogenization and centrifugation, and thereafter exhausted with 800 cc. of 1 *M* NaCl containing 0.01 *M* sodium citrate by shaking for 18 hours. The centrifuged supernatant was poured into 6 volumes of distilled water. The fibers of DNP carried down were purified as when Prep. 4 of the spleen DNA was prepared, and after dissolution in 200 cc. of 10% NaCl, poured into 2 volumes of 95% alcohol. The crude DNA thus given was deproteinized, precipitated, washed and dried as in the case of Prep. 1 of the splenic DNA. Yield 170 mg.

Properties of the Preparations

Composition

The preparations were dried to a constant weight in vacuo over P_2O_5 (room temperature) prior to analysis. As shown in Table I, the preparations of the placental DNA were contaminated with 4–5% PNA calculated as yeast PNA from ribose contents, whereas those of the splenic

DNA were fairly pure. Hence a part of the former preparations were freed from PNA by alkaline treatment on the principle of Wyatt[13] as follows. They were dissolved in 1 *N* NaOH, stood at 37°C for 18 hours, and after neutralization with glacial acetic acid, dialyzed. The solutions were precipitated with 1.5 volumes of 99% alcohol containing some NaCl and washed with alcohol and dried. The analytical figures of bases in the placental DNA in Table II concern the products thus given. Analysis of 5-methylcytosine was made according to Wyatt[14] with modifications :— All the paper chromatograms (first and second) were developed on Toyo Roshi filter paper No. 3 by ascending irrigation with a mixture of 70 cc. of isopropylalcohol, 17 cc. of conc. HCl (sp. gr. 1.18) and 13 cc. of water. 5-Methylcytosine emigrated with an Rf of 0.52. The eluates of the zones after the second irrigation were ascertained by the aid of their ultraviolet spectra that they contained no other kinds of base, and the extinctions at the wave-length of 283.5 mμ were converted into weights of 5-methylcytosine.

As shown in Tables I and II, no significant difference existed between the two DNAs as to composition excepting the content of 5-methylcytosine which, however, was determined only in one preparation each of the DNAs.

TABLE I

Composition of the DNAs from Human Spleen and Placenta

Source of DNA	Preparation No.	N* %	P† %	N/P	Diphenylamine reaction of deoxyribose[16] $E_{1cm}^{0.05\%}$ (λ 595 mμ)	Cysteine reaction of deoxyribose[17] $E_{1cm}^{0.1\%}$ (λ 490 mμ)	Dische value[18] ‡	PNA§ %	$\varepsilon(P)_{max}$ in water//
Human spleen	1	13.29	8.23	1.62	0.930	0.760	103	0.7	6420
	2	13.00	8.36	1.56	0.970	0.806	100	0.0	6230
	3	13.79	8.56	1.61	0.937	0.755	95	0.4	6420
	4	13.49	8.10	1.67	0.955	0.811	101	0.2	6650
	5	12.89	8.01	1.61	0.957	0.780	103	1.7	6800
Human placenta	1′	13.40	8.19	1.64	0.852	0.744	90	4.7	6020
	2′	12.95	8.07	1.61	0.860	0.720	92	3.8	6770
	3′	13.05	8.06	1.62	0.892	0.736	94	4.0	6980
	4′	13.43	8.43	1.59	0.957	0.786	98	3.9	6440
	5′	13.72	8.10	1.69	0.851	0.740	91	5.2	5940

* Micro Kjeldahl. † Plimmer method[19]. ‡ The color value of Prep. 2 which appeared most pure among the other preparations was designated 100 units. § Ribose was estimated by a cysteine method of Dische[20] and expressed in terms of free yeast PNA E. Merck. // pH of the solutions 6.0.

Optical properties

Ultraviolet absorptivity. $\varepsilon(P)_{max}$ values (atomic extinction coefficients with respect to phosphorus) were measured after addition to 4–6 mg%

TABLE II

Contents of Bases in Preparations of DNAs from Human Spleen and Placenta

Source of DNA	Preparation No.	In per cent					In molecular ratios§					Number of moles of total bases per 100 moles of P
		Adenine†	Guanine†	Cytosine†	Thymine†	5-Methyl-cytosine‡	Adenine	Guanine	Cytosine	Thymine	5-Methyl-cytosine	
Human spleen	1	8.9	7.9	5.8	8.7	0.32	12.6	9.9	10.0	13.0	0.47	91.2
	3	10.0	7.7	5.7	9.3		14.4	9.9	10.0	14.4		90.7
	5	9.3	7.5	5.4	8.4		14.1	10.2	10.0	13.7		90.7
Human placenta	3'*	9.5	7.7	5.6	8.8		14.0	10.1	10.0	13.9		//
	4'*	9.1	7.2	5.4	8.5	0.18	13.8	9.8	10.0	13.9	0.30	
	5'*	9.5	7.4	5.6	8.7		13.9	9.7	10.0	13.7		

* The preparation was freed from PNA and analyzed (See the text). † Masamune and Sakamoto's technique[21] was applied, and for calculation were used the molecular extinctions in 0.1 *N* HCl: adenine, 13.0×10^3 (λ 260 mμ)[13]; guanine, 11.0×10^3 (λ 250 mμ)[13]; cytosine, 10.0×10^3 (λ 276 mμ)[22]; thymine, 7.95×10^3 (λ 265 mμ)[13]. ‡ Modified Wyatt method (See the text) was applied and molecular extinction in 0.1 *N* HCl of 9.79×10^3 (λ 283.5 mμ)[22] was used for calculation. § The number of molecules of cytosine was assumed as 10 units. // Was not calculated because the analyses were made on substances previously treated with alkali (See the text).

solutions of the DNAs in water of an equal volume of water, 0.02 *N* HCl, 0.02 *N* NaOH or 12 *N* HCl. The spectral data of the solutions in 6 *N* HCl were determined 30 minutes after preparation of the solutions. The $\varepsilon(P)_{max}$ values of the watery solutions ranged from 6,000 to 7,000 in either of the splenic and placental DNAs (Table I). The ratios of $\varepsilon(P)_{max}$ values of acidic and alkaline solutions to the $\varepsilon(P)_{max}$ values of the corresponding watery solutions ($R\varepsilon(P)_{max}$) were found specific to preparation and well reproducible contrary to $\varepsilon(P)_{max}$ values of the watery solutions. Table III shows that the ratios of $\varepsilon(P)_{max}$ values of the splenic-DNA preparations in 0.01 *N* NaOH to the corresponding values of the same preparations in water were higher than the corresponding ratios in the placental DNA preparations, suggesting a higher polymerization grade of the former DNA as compared with the latter. Fig. 1 shows the spectra of Prep. 2 of the splenic DNA in the various solvents.

Next, one preparation each of the DNAs were examined regarding the effect of heating on their ultraviolet absorptivity. Portions of 3.6 mg% solutions in 0.01 *M* NaCl of Prep. 5 of the splenic DNA and of Prep. 3 of the placental DNA were heated for 10 minutes at various temperatures between 30 and 100°C inclusive at intervals of 10 degrees, and after

TABLE III

$\varepsilon(P)_{max}$ and $\varepsilon(P)_{min}$ Values of DNA Solutions (2–3 mg%) in Different Solvents as Expressed in Ratios $R\varepsilon(P)_{max}$ and $R\varepsilon(P)_{min}$, $\varepsilon(P)$ Values of the Solutions in Water Being Designated 1 Unit

Source of DNA	Preparation No.	Solvent			
		Water (pH 6.0)	0.01 *N* HCl	0.01 *N* NaOH	6 *N* HCl
			$R\varepsilon(P)_{max}$		
Human spleen	1	1 (λ_{max} 258–260 mμ)	1.31 (λ_{max} 260–262 mμ)	1.29 (λ_{max} 258–262 mμ)	1.54 (λ_{max} 264 mμ)
	2	1	1.34	1.23	1.50
	3	1	1.27	1.18	1.46
	4	1	1.30	1.03	1.40
	5	1	1.23	1.33	1.50
Human placenta	1′	1 (λ_{max} 258–259 mμ)	1.24 (λ_{max} 260–263 mμ)	1.15 (λ_{max} 258–260 mμ)	1.70 (λ_{max} 260–264 mμ)
	2′	1	1.27	1.14	1.48
	3′	1	1.30	1.02	1.47
	4′	1	1.37	1.10	1.44
	5′	1	1.36	1.20	1.60
			$R\varepsilon(P)_{min}$		
Human spleen	1	1 (λ_{min} 231–233 mμ)	1.10 (λ_{min} 233 mμ)	1.73 (λ_{min} 232–234 mμ)	1.82 (λ_{min} 230–233 mμ)
	2	1	1.17	1.70	1.86
	3	1	0.87	1.34	2.00
	4	1	1.04	1.43	1.72
	5	1	1.00	1.60	1.80
Human placenta	1′	1 (λ_{min} 230–232 mμ)	1.09 (λ_{min} 232–234 mμ)	1.19 (λ_{min} 232–234 mμ)	1.82 (λ_{min} 232–235 mμ)
	2′	1	0.89	1.32	1.88
	3′	1	1.00	1.44	2.42
	4′	1	1.20	1.32	1.85
	5′	1	1.35	1.41	2.42

cooling under running water, examined spectrophotometrically. As shown in Fig. 2, both of the DNAs underwent no change of absorptivity when heated at 60°C and lower temperatures. When heated at 70°C and higher temperatures, their absorptivities were elevated more and more with increase of temperature and that almost in parallel with each other. Furthermore, heating for a longer time than 10 minutes at a temperature raised the absorptivities no more. So the two DNAs must have nearly the same number of hydrogen bonds, which are split at each of temperatures 70, 80 and 90°C. The changes of absorptivity of the placental DNA by heating at 90 and 100°C were similar while the absorptivity of the splenic DNA was raised higher at 100°C than at 90°C.

Optical rotation. Solutions (pH 6.0) of the DNAs (sodium salts) in water were not perfectly clear so that the centrifuged supernatant were applied for the optical measurement which was effected in a 2.2 dm. tube. As shown in Table IV, the splenic DNA is more dextrorotatory

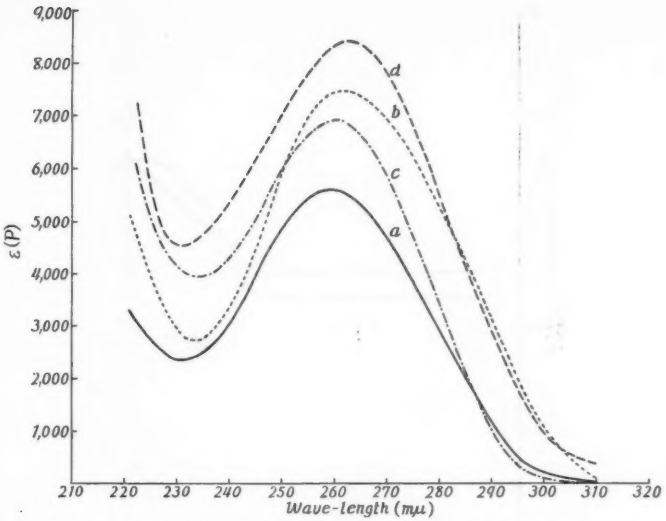

Fig. 1. Absorption spectra of 2 mg% solutions of Prep. 2 of the splenic DNA in water (*a*), 0.01 *N* HCl (*b*), 0.01 *N* NaOH (*c*) and 6 *N* HCl (*d*). Cell 1 cm. thick.

than the placental.

Viscosity

Relative viscosities of watery solutions were measured on Prep. 4 of the splenic DNA and Prep. 5′ of the placental DNA which looked to be most polymerized among the preparations of the respective DNAs. Their 112 mg% solutions were diluted progressively and examined in an Ostwald type viscosimeter of Neurath *et al.*[15] at 25°C. The latter preparation was obtained with placentas immediately after delivery, but the former with spleens after autopsy which was conducted 24 hours after death of the individuals. As illustrated in Fig. 3, however, the solutions of the placental-DNA preparation of concentrations higher than 40 mg% were less viscous than the corresponding solutions of the splenic-DNA preparation.

When considered in conjunction with the findings regarding the absorptivities, the placental DNA is most probably less polymerized than the splenic DNA, although, in the placental DNA, possibility of degradation during isolation due to DNAase, which seems to be rich in this organ, is not excluded.

Skin reaction of the DNAs

Preps. 4 and 5′ were dissolved in 1500 volumes of 0.85% NaCl

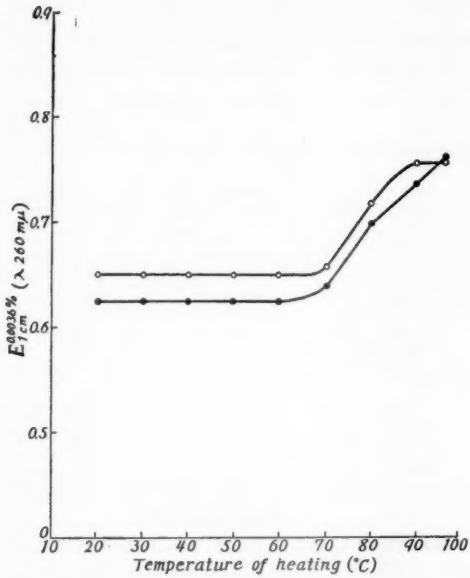

Fig. 2. Showing the effects of heating at various temperatures on absorptivity of 3.6 mg% solutions in 0.01 *M* NaCl of the splenic DNA (Prep. 5) (●) and of the placental DNA (Prep. 3) (○).

TABLE IV

$[\alpha]_D$ Values of the DNAs in Water

Spleen DNA		Placenta DNA	
Prep. No.	$[\alpha]_D$	Prep. No.	$[\alpha]_D$
3	+141° (t 12°; c 0.1)	1′	+105° (t 12°; c 0.2)
4	+124° (t 12°; c 0.1)	5′	+111° (t 12°; c 0.1)

containing 0.5% phenol, and 0.1 cc. portions of the solutions were injected intracutaneously on the flexor side of forearm. And after 4 hours, the longest and shortest diameters of the reddened areas were measured to calculate their averages. Fig. 4 indicates that the placental DNA is inclined to give rise to a bigger redness than the splenic.

Summary

1. Deoxyribonucleic acids were isolated as sodium salts from human spleen and placenta in a state as pure and highly polymerized as possible, and compared with each other.

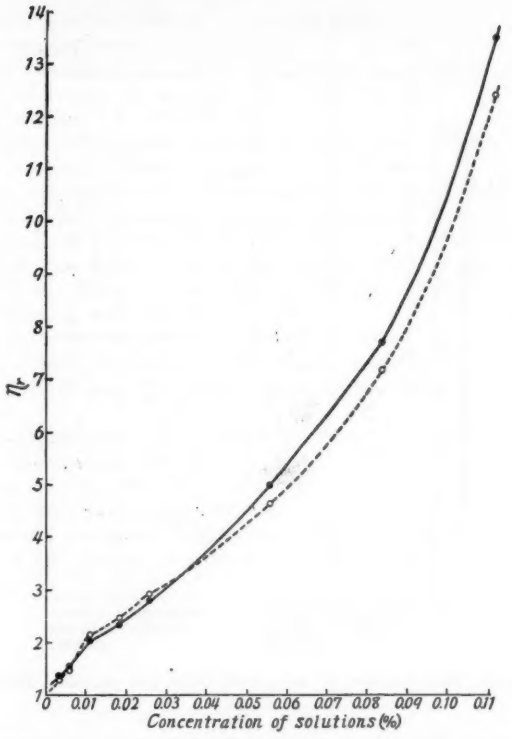

Fig. 3. Relative viscosities of the watery solutions of the splenic DNA (Prep. 4) (●) and of the placental DNA (Prep. 5′) (○). Temperature 25°C.

2. Although the splenic DNA preparations were mostly not contaminated with more than 1% pentosenucleic acid, those from placentas contained even 4–5% pentosenucleic acid.

3. The DNAs resemble each other in composition.

4. Although they gave similar values of $\varepsilon(P)_{max}$ in water which show poor reproducibility, the placental-DNA preparations gave definitely lower $\varepsilon(P)_{max}$ values than the splenic-DNA preparations in 0.01 *N* NaOH as against the corresponding values of the respective DNA preparations in water.

5. The both DNAs became more absorptive when heated in water at 70, 80 and 90°C. The higher the temperature of heating was, the higher was elevation of the absorptivities. The placental DNA showed the same elevation at 90 and 100°C contrary to the splenic DNA, which

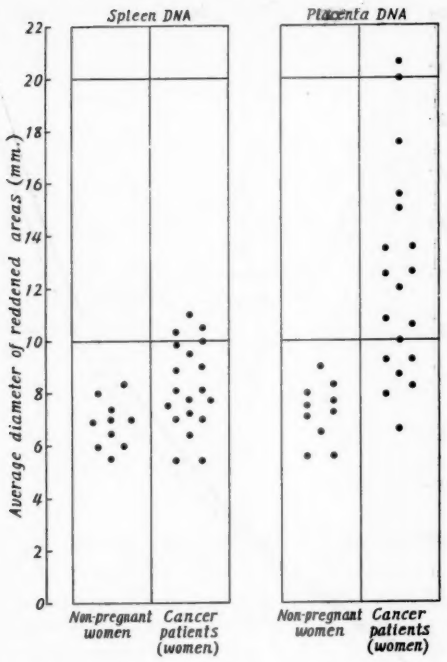

Fig. 4. Skin reaction of the splenic DNA and placental DNA.

became still more absorptive at the latter temperature than at the former.

6. The placental DNA gave less viscous solutions than the splenic DNA.

The findings in 4. to 6. above suggest a lower polymerization grade of the placental DNA than that of the splenic DNA.

7. The placental DNA is less dextrorotatory than the splenic DNA.

8. The placental DNA shows more intensive skin reaction in cancer patients than the splenic.

Thanks are due to the Ministry of Health and Welfare for a grant in aid to us, which is gratefully acknowledged. H. Masamune.

References and Notes

1) Masamune, Tsuiki, Kitabatake & Sakamoto, Tohoku J. Exp. Med., 1955, **61**, 171.
2) Oh-Uti, Ogawa, Yabuki & Kawasaki, *ibid.*, 1952, **56**, 177.
3) Ogawa, *ibid.*, 1952, **56**, 185.
4) Masamune & Ogawa, *ibid.*, 1952, **56**, 269.
5) Masamune, Kanno & Kimura, *ibid.*, 1954, **60**, 323.

6) Sato, Nihon Naikagakkai Zasshi (Jap.), 1956, **44**, 78.
7) Masukawa, Tohoku J. Exp. Med., 1953, **58**, 271.
8) Masamune, Yosizawa & Masukawa, *ibid.*, 1953, **58**, 381.
9) Chargaff, Experientia, 1950, **6**, 201 ; J. Cell. & Comp. Physiol., 1951, **38**, 41.
10) Chargaff & Lipshitz, J. Am. Chem. Soc., 1953, **75**, 3658.
11) Woodhouse, Bioch. J., 1954, **56**, 349.
12) Luck (Cold Spring Harbor Symposia on Quantitative Biology, 1950, **14**, 127) recommended for preparation of DNP from rat liver to preliminarily treat the tissue with 0.4 *M* NaCl than with 0.14 *M* NaCl with the aim of removing DNP. However, the writer found that DNAs are yielded less from the present sources and contaminated more with PNA when 0.4 *M* NaCl is used instead of 0.14 *M* NaCl, and could obtain from the tissues treated with 0.14 *M* NaCl preparations of the DNAs as pure as those from the PNPs of these tissues prepared according to Mirsky and Pollister (J. Gen. Physiol., 1946, **30**, 117).
13) Wyatt, Bioch. J., 1951, **48**, 584.
14) Wyatt, *ibid.*, 1951, **48**, 581.
15) Neurath, Cooper & Erickson, J. Biol. Chem., 1941, **138**, 411.
16) Chargaff & Davidson, The nucleic acids, Academic Press, New York, Vol. 1, 1955, pp. 287 and 303.
17) Stumpf, J. Biol. Chem., 1947, **169**, 367.
18) Dische, Mikrochemie, 1930, **8**, 4.
19) Plimmer, Bioch. J., 1933, **27**, 1810.
20) Dische, J. Biol. Chem., 1949, **181**, 379.
21) Masamune & Sakamoto, Tohoku J. Exp. Med., 1955, **61**, 353.
22) Shugar & Fox, Biochim. et biophys. Acta, 1952, **9**, 199.

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Nucleic Acids

IX. Chemical Comparison between DNAs of Human Placenta and of Human Spleen

Second Report: Regarding Partial Degradation Products

By

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In the First Report[1] the writer informed that the DNAs of human placenta and of human spleen closely resemble each other in composition but differ in polymerization grade and optical rotation. The present account deals with their acid-degradation products such as "pyrimidine-neucleic acid," which is similar to apurinic acid of Chargaff[2)-4)], and "thymic acid" of Kossel.[5)]

Experiments and Comments

Materials

The splenic and placental DNAs were prepared as Preps. 4 and 5′ in the First Report were, and the corresponding products after many runs were put together according to source. The mixtures analyzed as shown in Table I.

Degradation of the DNAs

Human splenic DNA

Prep. 6. This was prepared in a way most drastic among the preparations obtained by HCl-treatment. 300 mg. of the splenic DNA were dissolved in 74 cc. of distilled water, and after addition of 26 cc. of 0.1 *N* HCl to 0.026 normality, stood at 37°C for 24 hours. The digestate was dialyzed in a cellophane bag for 4 hours against running water at below 5°C and then for 24 hours against 1.5 *l.* portions of distilled water at below 10°C until it showed pH 6, and some precipitate that occurred in the mean time was rejected after centrifugation. The clear fluid was vacuum-distilled at below 37°C to 30 cc. and added to with 4 volumes of 99% alcohol and a few drops of the sat. NaCl (in water), whereby a

TABLE I

Composition of the Splenic and Placental DNAs Used as Materials for Acid-degradation

Methods of analysis were the same as those described in the First Report.

Source of DNA	Percentage							Diphenylamine reaction $E_{1cm}^{0.05\%}$ (λ 595 mμ)	Dische value‡
	N	P	Adenine	Guanine	Cytosine	Thymine	PNA†		
Human spleen	13.3	8.3	9.3	7.2	5.6	8.8	1.2	0.952	99
Human placenta*	13.4	8.2	9.5	7.7	5.5	8.6	4.9	0.876	92

* The analysis of purines was made on a specimen freed from PNA as described in the First Report. † Calculated as free yeast PNA from the ribose contents as in the First Report. ‡ The color value of Prep. 2 in the First Report is assumed as 100 units.

flocculent precipitate was given. After standing in a refrigerator overnight, the deposit was centrifuged off, washed with 66 (w/v), 99 (commercial) and 100% alcohol in turn and dried in vacuo over H_2SO_4. Yield 155 mg.

Preps. 1–5. These were prepared under milder conditions than above, as illustrated in Table II, in which the products are arranged in the order from mild to rough treatment for preparation. Besides, the hydrolysates were neutralized before dialysis, excepting the case of Prep. 4.

TABLE II

Conditions for HCl-degradation of Spleen DNA and Yields of the Products

Prep. No.		1	2	3	4	5	6
Weight of material used (mg.)		110	110	120	160	200	300
HCl for degradation	Normality	0.01	0.01	0.01	0.02	0.022	0.026
	Volume (cc.)	60	70	70	103	67	100
Duration of acid treatment (hrs.)		6	10	24	24	27	24
Yield of products (mg.)		85	86	81	110	116	155

Prep. 7 (barium thymate). It was prepared by a modification of Bredereck and Müller's method[6] as follows. 500 mg. of the splenic DNA were dissolved in 50 cc. of distilled water, heated to 80°C in a water-bath, and after addition of 6 cc. of 2 *N* H_2SO_4, maintained at the same tempera-

ture for 30 minutes. A white, gelatinous deposit that appeared first dissolved into an opalescent solution. This solution was added to with 170 mg. of a $HgSO_4$-powder and shaken at 80°C for 15 minutes and at room temperature for subsequent 30 minutes, followed by centrifugation. The transparent supernatant was mixed with 4.8 cc. of a solution containing 0.35 g. of $BaCl_2 \cdot 2H_2O$ and 2.26 g. of $Ba(AcO)_2 \cdot H_2O$. The abundant white precipitate was centrifuged off, and the clear supernatant was placed in a refrigerator overnight after addition of 4 volumes of 99% alcohol. The occurring deposit was washed with 90, 99 and 100% alcohol and dry ether in succession and dried in vacuo at 76.7°C over P_2O_5. Yield 151 mg.

Human placental DNA

Prep. 6'. 300 mg. of the placental DNA were subjected to the strictly same procedure as Prep. 6 from the splenic DNA was. Yield 177 mg.

Prep. 7'. This was prepared as Prep. 7 from the splenic DNA, starting with the same amount (500 mg.) of material, and 138 mg. were yielded.

Properties of the Degradation Products

All the preparations dissolved in water transparently, but Preps. 1 and 2 precipitated from their watery solutions on addition of HCl to a pH lower than 1.5, whereas the others did not. Most of the analyses were processed by the methods employed in the previous work.[1] Schiff test was carried out by the quantitative-analytical procedure which will be described in the Third Report.[7] Barium was determined gravimetrically as $BaSO_4$. Ultraviolet absorptions were measured in a 1 cm. thick cell on 4 mg% solutions in water (pH of the solutions 6.0), 0.01 *N* HCl, 0.01 *N* NaOH and 6 *N* HCl of the preparations, and optical rotations in a 1 dm. tube on 1% solutions in water (pH of the solutions 6.0). The figures given are embodied in Table III.

Preps. 3–6 (from the splenic DNA) resemble apurinic acid of Chargaff[2)–4)] in composition. However, they have been obtained under milder conditions. Although traces of purines were detected in them, those are ascribed to the ribonucleic acid contaminating the starting materials (Cf. the First Report[1]). Diphenylamine reaction showed a weak trend that the stronger the acid-treatment was, the poorer was the deoxyribose content of the product. The strengths of Schiff reaction of the preparations nearly equal one another, when expressed in extinction ratios of the color developed thereby to that developed by the diphenylamine reaction. In Preps. 1 and 2, in which no small amounts of purines still remain undetached, the latter preparation, whose Schiff reaction was more intensive, contains more of phosphorus and less of nitrogen and purines. So Prep.

TABLE III

Properties of the Acid-degradation Products of the Splenic and Placental DNAs

Source of DNA		Human spleen							Human placenta	
Prep. No.		1	2	3	4	5	6	7	6′	7′
Diphenylamine reaction $E^{0.05\%}_{1cm}$ (*a*)		1.188	1.179	1.202	1.175	1.079	1.065	0.826	1.019	0.845
Schiff reaction $E^{0.004\%}_{1cm}$ (*b*)		0.249	0.332	0.628	0.570	0.534	0.550	0.390	0.565	0.398
$\frac{b}{a}\times 10$		2.10	2.82	5.23	4.90	4.96	5.16	4.72	5.54	4.71
N %		11.44	10.27	5.91	5.49	5.22	6.15	6.10	5.16	5.05
In per cent	P	8.56	8.92	10.54	10.07	10.51	10.14	8.96	10.19	8.73
	Adenine	6.3	5.3	0.2	0.2	0.3	0.5	0.3	0.6	0.3
	Guanine	4.9	4.7	0.5	0.4	0.3	0.5	0.4	0.6	0.3
	Cytosine	5.4	5.5	6.9	6.7	7.1	7.3	5.8	6.4	6.1
	Thymine	8.7	8.6	11.6	10.5	11.1	11.2	10.2	10.5	9.9
In molecular ratios*	P	100	100	100	100	100	100	100	100	100
	Adenine	16.8	13.8	0.4	0.4	0.7	1.1	0.7	1.4	0.8
	Guanine	11.9	11.0	1.2	0.8	0.6	1.0	0.9	1.3	0.7
	Cytosine	17.7	17.4	19.6	18.9	18.8	20.1	18.0	17.5	19.5
	Thymine	24.8	24.0	28.0	26.1	25.9	27.3	28.0	25.2	27.9
Total bases per 100 moles of P (moles)		71.2	66.2	49.2	46.2	46.0	49.5	47.6	45.4	48.9
$\frac{\text{Thymine}}{\text{Cytosine}}$ (mol. ratio)		1.40	1.38	1.43	1.38	1.38	1.36	1.56	1.44	1.43
Ba %								22.94		21.58
$[\alpha]^{13}_{D}$ (water) (degree) (*c*)		67	57	42	40	34	30	25	54	32
$\frac{c}{a}\times 10$		5.64	4.84	3.49	3.40	3.15	2.95	3.03	5.29	3.79
$E^{0.004\%}_{1cm}$ in water (λ_{max} in mμ)		0.820 (262)	0.780 (262)	0.593 (266)	0.593 (267)	0.598 (267)	0.621 (266)	0.601 (266)	0.476 (266)	0.521 (266)

* P is designated 100 units.

3 is the one whose deoxyribose is least destroyed among the purine-free products. It is named "sodium pyrimidinenucleate." Pyrimidine/P and cytosine/thymine ratios vary scarcely within all of Preps. 1–6. The

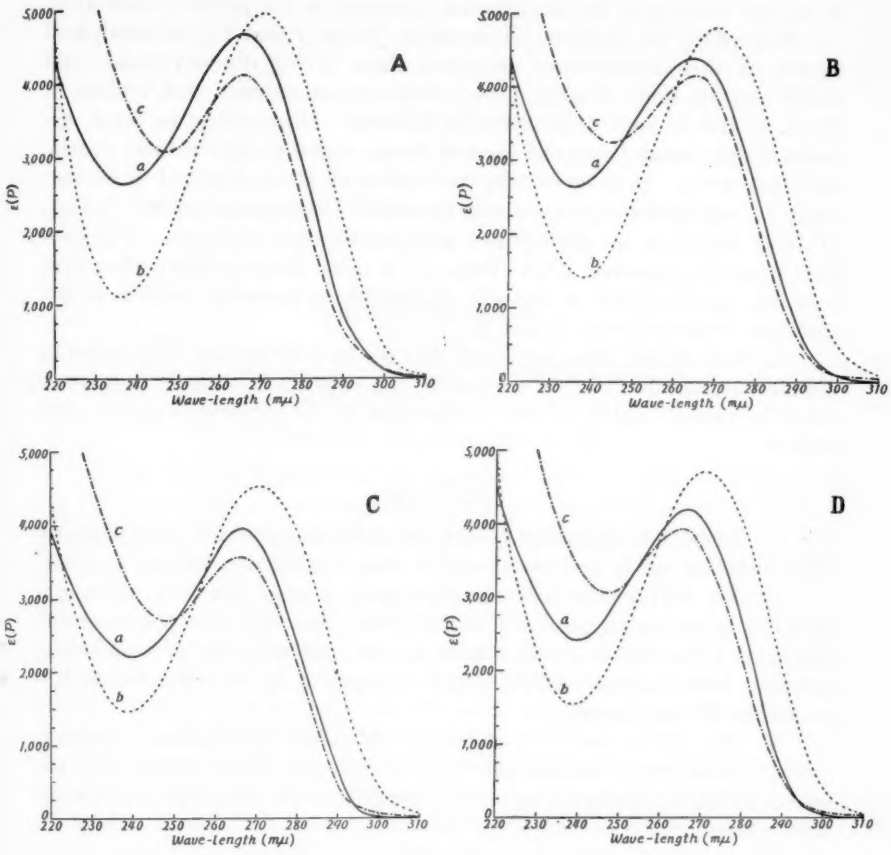

Fig. 1. Absorption spectra of 4 mg% solutions of Preps. 6 (**A**), 6′ (**B**), 7 (**C**) and 7′ (**D**) in water (pH of the solutions 6.0) (*a*), 0.01 *N* HCl (*b*) and 0.01 *N* NaOH (*c*).

wave-length of maximum absorption shifted towards the longer with progress of degradation from Prep. 1 to Prep. 3. Absorption spectra of Prep. 6 in water, 0.01 *N* HCl and 0.01 *N* NaOH are shown in Fig. 1A. Optical rotation decreased suddenly from Prep. 1 to 3 and gradually from Prep. 3 to 6.

Prep. 6′ from the placental DNA rotated D-light to the right decidedly more than Prep. 6 the corresponding preparation from the splenic DNA, in distinction from similarity in other respects of them (See Table III and Figs. 1A and 1B). It is noteworthy that the DNAs themselves are

in inverse relation to the degradation products in the present connection.

Regarding the thymate preparations (Preps. 7 and 7′), the analytical figures of their components excepting those giving diphenylamine- and Schiff-reaction don't diverge from calculation as assumed that sodium of Preps. 6 and 6′ were replaced with barium. Deoxyribose in them appears slightly more destroyed than in Preps. 6 and 6′ by the more drastic acid-treatment. In other words, the residue of DNA deprived of purines must be regarded as pretty stable to acids. Absorption spectra in Figs. 1C and 1D show no discrepancy between the two thymates. The product from the placental DNA (Prep. 7′) is more dextrorotatory than that from the splenic DNA as the sole distinguishing property, which is the case also between Prep. 6′ and 6.

In short, it has been suggested that the two DNAs are constituted of different pyrimidine nucleates, that is, the two DNAs diverge from each other in mutual union of the components in the pyrimidinenucleic acid moiety.

Summary

1. The DNAs of human spleen and of human placenta were partially degraded with acids and the products were compared with one another.

2. No discrepancy was found in most respects between the corresponding degradation products of the DNAs, but two products from the placental DNA rotate D-light more to the right than the corresponding products from the other DNA, which is opposite to the relationship between the DNAs themselves.

3. The DNAs are fairly stable to acids after elimination of purines.

4. Thus it has been found that the placental DNA differs from the splenic DNA in mutual linkage of the components in the pyrimidinenucleic acid moiety.

The Ministry of Health and Welfare gave a grant in aid to us, which is gratefully acknowledged. H. Masamune.

References

1) Sakamoto, Tohoku J. Exp. Med., this volume, p. 269.
2) Tamm, Hodes & Chargaff, J. Biol. Chem., 1952, **195**, 49.
3) Tamm, Shapiro, Lipshitz & Chargaff, *ibid.*, 1953, **203**, 673.
4) Tamm & Chargaff, *ibid.*, 1953, **203**, 689.
5) Kossel & Neumann, Z. physiol. Chem., 1896–97, **22**, 74.
6) Bredereck & Müller, Ber. dtsch. chem. Ges., 1939, **72**, 115.
7) Sakamoto, Tohoku J. Exp. Med., in press.

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Nucleic Acids

X. Chemical Comparison between DNAs of Human Placenta and of Human Spleen

Third Report: Schiff Reaction of the DNAs during the Course of Acid Hydrolysis

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(Received for publication, July 10, 1956)

In the Second Report,[1] it was suggested that pyrimidinenucleates of the DNAs from the two sources are of different structures despite of similar compositions. If then, the purines of the placental DNA might be cleft off by acids more rapidly or more slowly than those of the splenic DNA, because hydrolysis constant of glycosides depends upon the structure of aglycon as well as upon that of the sugar moiety even among α- or β-glycosides. In the present case, pyrimidinenucleate represents the sugar moiety. To follow up the course of the hydrolysis, the writer adapted Schiff reaction as a measure of free aldehyde group corresponding to deoxyribose whose glycosidic link is cleft.[2]

Solutions of DNAs in water or in 10% NaCl become Schiff-positive by mere dialysis, and Stacey[3]-[5] ascribed it to fission of labile glycosidic phosphate links which partake in polymerization of DNA, being based on the fact that inorganic phosphorus appears in the dialysate simultaneously. However, the writer found with a quantitative-analytical method which will be described below that the amount of deoxyribose residues reacting with Schiff reagent in a dialyzed DNA (Experimented on Prep. 4 of the splenic DNA in the First Report) only reaches 3–4% of the maximum value when the DNA is hydrolyzed with a mild acid to set free all the purines. Judging from the very weak union between purines and the remaining part of DNAs as has been revealed in preparation of a pyrimidinenucleate (See the Second Report[1]), it seems more plausible to suppose that DNAs turn Schiff-positive in dialysis owing to detachment of a tiny part of purines by the faint acidity of water. Although purines are not detected in the outer fluid, such traces of purines as are liberated in that way may be retained in the inner fluid through interaction between

them and the negatively charged residual part of the DNA, and the phosphorus coming out into the dialysate is possibly a contamination of the DNA preparation. Apart from this, even if the opinion of Stacey hold true, deoxyribose glycosidically bound with phosphoric acid can occupy only a small percentage of the total deoxyribose and is neglected in the argument below.

Experiments and Comments

Quantitative Schiff Reaction

In 1928, Widström[6] scrutinized the conditions under which Schiff reaction can be used for estimation of DNAs. Recently, however, Overend[7] found that the extinction of the color developed does not parallel the amount of deoxysugars, and so the reaction remains unutilized as a quantitative method. Fortunately, the writer proved that the extinctions follow Lambert-Beer law, if reaction mixtures are stood in an ice-chest until they show constant extinctions. The devised method which is a modification of Widström's is as follows:—

Schiff reagent. A 0.4% fuchsine (Fuchsin kleine Kristalle E. Merck) solution is bubbled with SO_2 gas until it turns straw-yellow, and then aerated to remove the irritative smell. It is preserved in a stoppered brown bottle at below 5°C. This reagent should show a pH of 4 or more when diluted twenty times with water.

The technique. An accurate quantity from 0.8 to 8 mg. of a partial degradation product of a DNA is dissolved in 100 cc. of water, and 4 cc. of the solution is pipetted into a stoppered (ground joint) test tube, added to with 0.2 cc. of the Schiff reagent and stood at 26°C for 30 minutes at first and afterwards at 8–10°C for 80 minutes. Thereupon the extinction at the wave-length of 550 mμ is measured. The control must be always carried out in parallel employing a definite degradation product of a DNA, f. i., a sodium pyrimidinenucleate specimen, because the extinction varies with the SO_2-content of the reagent.

Fig. 1 shows an example on a sodium pyrimidinenucleate revealing that the extinction is a straight line function of the amount of the material analyzed.

Schiff Reaction of the DNAs after Various Durations of Acid Treatment

1) 40 mg. each of the DNAs in the First Report[8] (Preps. 4 (spleen DNA) and 4′ (placenta DNA)) were dissolved in 50 cc. of distilled water, made up to 100 cc. with 0.02 *N* HCl (pH of the solutions ca. 2.1) and stood in an incubator at 37°C. 2 cc. portions were pipetted out at in-

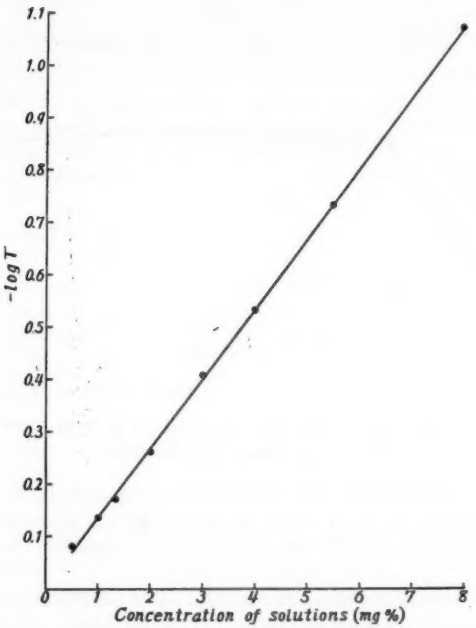

Fig. 1. Showing the correlation of extinction (Cell 1 cm. thick) in Schiff reaction with amount of substance on Prep. 6 from the splenic DNA in the Second Report.

TABLE I

Relation of Incubation Time with Schiff Reaction, when 40 mg% Solutions of the Splenic and Placental DNAs in 0.01 *N* HCl Are Stood at 37°C

Extinctions were measured in a 1 cm. thick cell, after neutralizing and diluting the solutions to 4 mg%.

Incubating time (hrs.)	Human spleen DNA	Human placenta DNA
	Extinction at λ of 550 mμ	
1	0.094	0.135
2	0.264	0.396
4	0.439	0.530
6	0.550	0.561
8	0.545	0.558
10	0.552	0.560
12	0.558	0.552
14	0.550	0.560
16	0.554	0.560
24	0.530	0.550
28	0.530	0.545
36	0.516	0.548
48	0.514	0.539

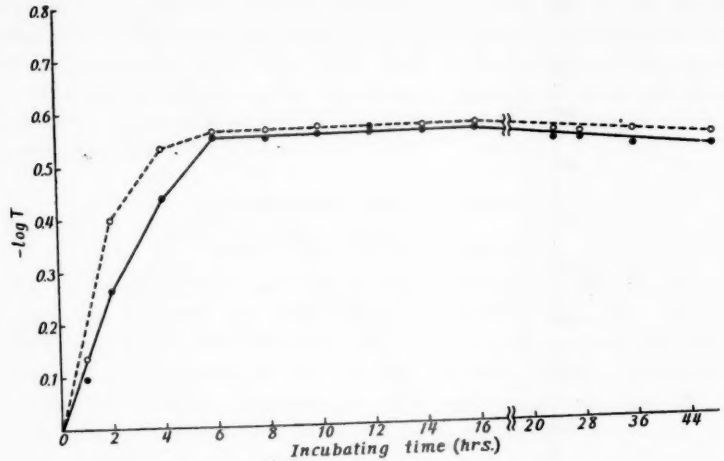

Fig. 2. Showing the increase of Schiff-positive residue with time when 40 mg% solutions in 0.01 *N* HCl of the splenic (●) and placental (○) DNAs were incubated at 37°C (Curves plotted from the figures in Table I).

tervals from the solutions, neutralized and after addition of water to 20 cc., examined as for the Schiff reaction. The solutions gave the maximum reaction after 6 hours from the commencement of incubation, and thereafter the reaction showed no noticeable alteration of strength within 16 hours. The maximum reactions of the solutions equaled each other. But, as shown in Table I and Fig. 2, the placental DNA gave rise to much stronger reaction than the splenic DNA in the period before the sixth hour, that is, purines were split off with a larger hydrolysis constant from the placental DNA than from the splenic. At the 24th hour of hydrolysis, the diphenylamine reaction of the solutions diluted to 40 mg% of the placental and splenic DNAs gave extinctions of 0.791 and 0.806 (at λ of 595 mμ) respectively, deoxyribose reactive to this reagent of the two solutions amounting nearly equal.

2) Two preparations each of the DNAs (Preps. 4, 5, 3′ and 5′ in the First Report) were taken, and 20 mg. portions of them were separately dissolved in 40 cc. of water. The solutions were adjusted with 0.1 *N* HCl to pH 4.0 a weaker acidity than in 1) and placed in a thermostat at 25°C for 20 hours. Then, they were neutralized with 0.1 *N* NaOH and made up to 50 cc. with water and their Schiff reaction was examined. The hydrolysates of the placental-DNA preparations gave larger E_{max} values than the splenic-DNA preparations, notwithstanding that the diphenylamine reactions of them were rather of strengths in reversed

TABLE II

Schiff and Diphenylamine Reactions of the DNAs Stood as 50 mg% Solutions of pH 4.0 for 20 Hours at 25°C

Extinctions in Schiff and diphenylamine reactions were measured in a 1 cm. thick cell after dilution of the neutralized solutions to 4 and 40 mg% respectively. The figures in parentheses are extinctions for the unhydrolyzed materials.

Source of DNA		Human spleen		Human placenta	
No. of preparation*		4	5	3′	4′
Extinction	Schiff reaction λ_{max} 550 mμ	0.542	0.540	0.625	0.671
	Diphenylamine reaction λ_{max} 595 mμ	0.760 (0.764)	0.758 (0.765)	0.700 (0.715)	0.769 (0.766)

* Preparations in the First Report.[8]

relation (Table II).

SUMMARY

1. Schiff reaction has been made up into a quantitative-analytical method for determination of deoxyribose with free carbonyl in degraded DNAs.

2. Occurrence of the Schiff-positive residue was followed up during mild acid hydrolyses of DNAs from human placenta and spleen.

3. The Schiff-positive residue was produced more quickly from the placental DNA than from the splenic DNA, in other words, purines in the placental DNA are less stably linked than those in the splenic DNA. After reaching the maximum, the intensities of the reaction of the DNAs were nearly the same within 16 hours and then decreased gradually. The maximum values equaled each other.

The expenses of this work were defrayed by a grant from the Ministry of Health and Welfare, which is gratefully acknowledged. H. Masamune.

References

1) Sakamoto, Tohoku J. Exp. Med., this volume, p. 281.
2) Tamm & Chargaff, J. Biol. Chem., 1953, **203**, 689.
3) Li, Overend & Stacey, Nature, 1949, **163**, 538.
4) Lee & Peacocke, J. Chem. Soc., 1950, 3361.
5) Overend, Stacey & Webb, *ibid.*, 1951, 2450.
6) Widström, Bioch. Z., 1928, **199**, 298.
7) Overend, J. Chem. Soc., 1950, 2769.
8) Sakamoto, Tohoku J. Exp. Med., this volume, p. 269.

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Studies of the Mechanics of Pulmonary Ventilation in Patients with Bronchial Asthma

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The chief complaint of asthmatic patient is dyspnea, but how this symptom occurs remains still considerably unknown.[1)-5)] Despite the accumulation of informations about pulmonary functions of asthmatic patients, many investigators[1),4),6),7),8)] have chiefly concerned the problems of lung volume, pulmonary gas distributions, ventilations and recently pulmonary circulations. Moreover, these investigators have took account only for studies in remissions or after administration of the drugs as adrenalin.

According to peculiarity of this disease, the physiological investigation of dyspnea in asthmatic patient during paroxysm may include possible importance concerning to the genesis of bronchial asthma. Although this sort of study should need certainly measurements of the pressure-flow relationship of the lung,[9)-25)] many have only attempted to obtain intrapleural pressure or flow rate at the mouth separately except Sheldon and Otis[3)] who reported intraalveolar pressure-resistance of bronchial canalization-flow rate relationships in remissions and after administration of epinephrine.

In previous study the present author observed evidently the diminution of vital capacity and one second vital capacity (or Tiffeneau's Index[7)]) of patients with bronchiectasis after inhalation of pilocarpin. Similarly in asthmatic patients spirograms change apparently 30–40 seconds after 1:100 pilocarpin inhalation, and characteristic asthma-like attacks are usually induced. The present study represents an attempt to explore functionally the behavior of the pulmonary tissues and bronchial trees during such asthma-like attack induced by pilocarpin inhalation.

Experimental

Experimental Procedure

8 patients with bronchial asthma clinically diagnosed and 5 normal

individuals have been studied. Table I demonstrates lung volumes, RV/TLC × 100, MBC, Tiffeneau's Indexes (one second vital capacity/vital capacity × 100) and anatomical dead spaces capable to indicate pulmonary gas distribution of these patients.

TABLE I

Results of Ventilatory Functions in 8 Asthmatic Patients

Subject Name	Sex	Age	Clinical diagnosis	TCL (L)	VC (L)	(RV/TCL ×100 (%)	MBC (L/min.)	Tiffeneau's index (%)	Anatomical dead space (cc.)
F.C.	♂	23	Bronchial asthma	5.60	4.03	28.0	106	65.5	178
Y.M.	♂	37	Bronchial asthma with pulm. emphy.	4.98	2.04	59.0	31	33.0 77.4	329
I.O.	♂	31	Bronchial asthma	4.93	3.44	30.2	92		—
K.M.	♂	48	" "	7.71	4.49	41.7	86	58.5	190
I.S.	♂	54	" "	6.25	4.09	34.5	79	62.0	240
Y.T.	♂	31	" "	5.42	3.83	29.3	85	63.3	149
F.K.	♀	28	" "	4.47	2.90	35.1	33	45.0	166
S.S.	♀	60	Bronchial asthma with pulm. emphy.	3.69	1.49	59.6	18	61.0	180

Volume changes were measured by Krogh's respirometer with the potentiometer which was connected to the galvanometer electrically. The speed of air flow was recorded at the mouth with a Fleisch's pneumotachograph connected to the differential manometer (Druckdifferentialkapsel). As a measurement of transpulmonary pressure the pressure difference between the mouth and the lower portion of the oesophagus was recorded. The intraoesophageal pressure was transmitted to one side of a differential manometer which was made identically to Fleisch's Druckdifferentialkapsel by means of air-filled polyethylene tube 60 cm. in length, 1.77 mm. in inside diameter, with about 50 holes in the catheter wall at the distal end.[15),17),22)] A thin-walled condome rubber balloon with a volume of 8 ml. undistended and 15 cm. in length was sealed to the catheter and enclosed the openings. The other side of the manometer membrane was connected to the mouthpiece chamber by pressure tubing. The air volume of the balloon during recording was 1 ml. In front of the mouthpiece and the pressure tubing a solenoid valve was attached for instantaneous electrical interruption of respiratory flow. Volume changes, flow rates and pressures were recorded simultaneously on a three elements oscillograph.

All patients were studied asymptomatic remissions. Preliminary the determination of the volume and timed vital capacity was carried out

utilizing the Knipping's and Krogh's respirometer in sitting position. Then the oesophageal balloon was swallowed. In some patients asthmatic attacks were provocated only by passing of the balloon through the nose so that the tracing of oscillogram was usually obtained 10 or more minutes after setting of the balloon into the oesophagus. Calculation was made from the data during resting and rapid breathing. After these procedures patients inhaled orally 1 :100 solution of pilocarpin by nebulizer for 30–60 seconds. Immediately after patients had complained breathlessness slightly as observing the pressure fluctuation of the oscillograph, the inhalation was stopped and [in established paroxyms oscillograms were obtained. The last recording was taken after inhalation of 1 :1000 adrenalin in the same way untill subjective symptom disappeared.

Calculation. The effective compliance of the lung[24] (L/cmH_2O) in this paper represents the relationship of lung volume change to the coresponding elastic pressure change at the instance of zero air flow of resting breathing and therefore it is usually smaller than the static compliance all of which does not effective even in normal subjects. The work used for elastic property of the lung (gm. L/L) is stored in the lung as potential energy inspiratorily, releasing it at expiration. The inherent limitation of the present experiment makes it impossible to obtain any absolute value of intraoesophageal pressure, so that the elastic work was calculated from volume change-intraoesophgeal pressure diagram assuming oesophageal pressure equals zero at the beginning of inspiration, and consequently trapezoid was reduced to a triangle in diagram. This assumption would seem to be sufficient for comparison.[23] The effective resistance ($cm.-H_2O/L/sec.$) is the relationship of the flow-resistive component of pressure difference between the mouth and the surface of the lung to the simultaneous rate of volume change of the lung, and equivalent to " viscance " of Bayliss & Robertson.[10] The maximal value of effective resistance in a single breathing cycle is the maximal resistance. The non-elastic work (gm. L/L) is required for flow-resistive properties of the lung and airways. All works were corrected to the work per liter ventilation for comparison.

Results with Discussion

Normal subjects

Results in normal subjects during quiet breathing are summarized in Table II. The effective compliances of normal lungs in 5 subjects were 0.157 $L/cm.H_2O$ on an average which seemed to be lower compared with that of previous observors. The maximal resistances were 2.1 and 2.8 cm./L/sec. in inspiration and expiration, respectively. The power factor was 0.47 resulting from 61° in mean phase angle. The mechanical properties of the lungs in 5 normal subjects may best be seen by referring

TABLE II

Mechnical Properties of the Lungs in 5 Normal Subjects

Subject	Effective compliance (L/cmH_2O)	Elastic work (gm.L/L)	Maximal resistance ($cmH_2O/L/sec.$)		Non-elastic work (gm.L/L)		Phase angle (°)	Power factor (Cos θ)
			Insp.	Exp.	Insp.	Exp.		
S.O.	0.148	3.4	2.6	3.2	0.7	0.5	56	0.56
N.S.	0.115	4.3	1.4	1.0	0.8	0.4	63	0.45
T.M.	0.234	2.1	2.1	2.2	0.4	0.3	58	0.53
K.S.	0.128	3.9	2.6	4.1	1.0	0.7	64	0.44
A.A.	0.160	3.1	1.7	3.7	0.5	0.6	68	0.37
Mean	0.157	3.3	2.1	2.8	0.7	0.5	61	0.47

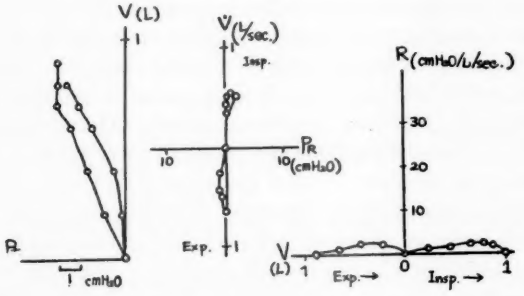

Fig. 1. Pressure-volume, pressure-flow rate and volume-resistance diagrams in normal subject. V : volume change, P_t : intraoesophgeal pressure, V̇ : flow rate, P_R : pulmonary pressure, R : resistance.

to diagrams in Fig. 1. In relationship of flow to pressure both inspiratory and expiratory curves described small loops in our experiments. The elastic energy stored in the lung inspiratorily was 0.33 gram L/L and seemed to be enough to perform the expiratory non-elastic work not using any other energy than potential energy stored in it.

Asthmatic patients between attacks

Table III represents datas of breathing mechanics in asthmatic patients during remissions. The effective complicance was reduced slightly, resulting in the increase of elastic work. The maximal resistance in quiet breathing cycle increased markedly and the non-elastic work was approximately 4-fold of that in normal subjects. But no consistent difference was found in inspiratory and expiratory effective resistance. The moderate increment of the power factor due to the diminution of phase angle was also observed.

TABLE III

Mechanical Properties of the Lungs in 8 Asthmatic Patients during Remissions

Name	Effective compliance ($L/cm.H_2O$)	Elastic work (gm.L/L)	Maximal resistance ($cmH_2O/L/sec.$)		Non-elastic work (gm.L/L)		Phase angle (°)	Power factor ($Cos\theta$)
			Insp.	Exp.	Insp.	Exp.		
F.C.	0.11	4.6	8.1	10.2	6.3	4.8	8	0.99
Y.M.	0.20	2.5	6.6	6.4	3.6	2.8	9	0.98
I.O.	0.10	5.0	3.4	4.3	1.2	1.1	52	0.61
K.M.	0.13	3.9	1.3	2.8	1.0	1.7	81	0.15
I.S.	0.11	4.6	2.2	5.0	0.8	1.2	52	0.61
Y.T.	0.06	8.3	8.7	12.2	1.7	2.0	36	0.80
F.K.	0.11	4.6	10.5	13.5	2.4	1.7	9	0.98
S.S.	0.04	12.5	11.0	15.5	3.6	2.5	24	0.91
Mean	0.11	5.7	6.4	8.7	2.5	2.2	33	0.75

These results indicate generally that asthmatic patients consume more energy for breathing than normal subjects even during remissions.

Asthmatic patients during attack

After 30–40 seconds of nebulization with 1:100 pilocarpin solution to asthmatic patients, asthma-like attacks were immediately and clearly provocated in nearly all cases. The tracing of the mouth-to-oesophagus pressure difference, rate of flow and volume change during voluntary ventilation are shown in Fig. 2. An upper tracing of a normal subject

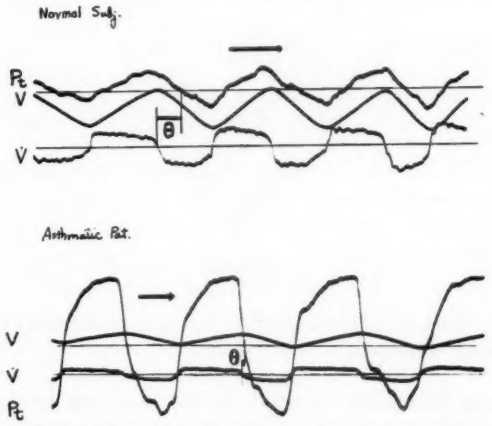

Fig. 2. Oscillograms during resting breathing in a normal subject and a asthmatic patient during attack. θ: phase angle.

is shown for comparison with the lower of an asthmatic patient during attack. Breathing patterns are strikingly different. Although asthmatic patient reduced his tidal volume as well as flow rate, the marked fluctuation of pressure curve in a single breathing cycle was characteristic. The obliteration of irregularities in the flow patterns seems to be due to the increase of resistance without any calculation of resistance. If we may consider that the pulmonary system is analogous to an electrical circuit consisting of a capacitor and a resistor connected in series, the current with high frequency will pass in this system more freely than the low frequency current. Accordingly, many irregularities appear in flow rate of such recordings as in normal subjects. On the other hand, in asthmatic patients the resistor is enough increased to neglect the effect of a capacitor so that the irregularities of flow patterns will disappear. There were no prolonged expirations in asthmatic patients.

Immediately after administration of nebulized pilocarpin, Vital Capacity and Tiffeneau's Index were extensively reduced except one who had complained no dyspnea, as seen in Fig. 3. These diminution recovered mostly after adrenalin inhalation. The effective compliance of the lung decreased considerably, especially in a patient with pulmonary emphysema (Fig. 4). According to the decrease of compliance, the elastic

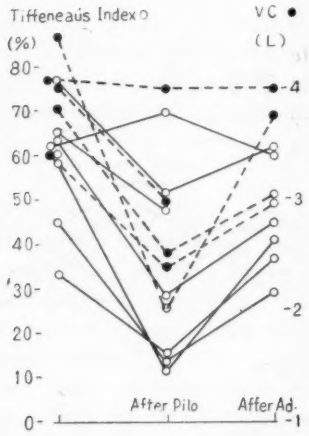

Fig. 3. Changes of Tiffeneau's index and vital capacity after pilocarpin and adrenalin inhalation.

work of the lung increased, in other words the lung became difficult to inflate. The striking increase in resistance of the lung and bronchial trees was also observed, resulting in greater work for frictional resistance

both inspiratorily and expiratorily. Any important differences of resistance between inspiration and expiration were not found, similarly in remissions.

These relationships are graphically shown more clearly in following diagrams in which transpulmonary or flow resistive pressure difference is on the abscissa and volume change or flow rate on the ordinate. Initially, pressure-volume curves represent the marked change in the shape of the loop which shows pronounced flatness in asthmatic attack (Fig. 5).

Fig. 4

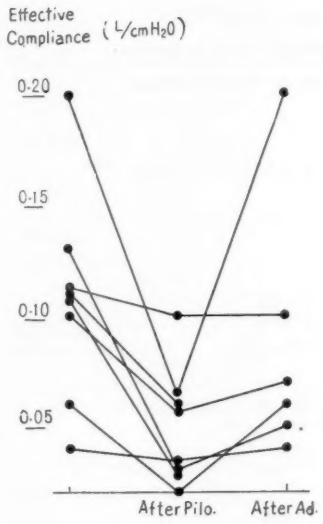

Fig. 5

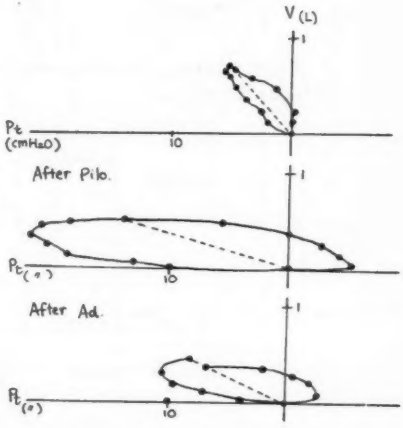

Fig. 4. Changes of the effective compliance after pilocarpin and adrenalin inhalation.

Fig. 5. Changes in pressure-volume graph of a asthmatic patient after pilocarpin and adrenalin inhalation.

This is undoubtedly due to the increase of resistance and may best be seen in pressure-flow graph (Fig. 6). These curves are not single lines but describe loops in both inspiration and expiration, although the latter recording little more large loops. The differences in volume-resistance graph between normal subjects and asthmatic patients are demonstrated in Fig. 7 with typical 3 cases. In remissions Patient K.M. showed normal resistance, which was increased strikingly after inhalation of pilocarpin, but adrenalin could not diminish the increased resistance to pre-pilocarpin value. In Pat. Y. M. with relatively high resistance in remissions the resistance recovered roughly to previous level after administration of adrenalin. Exceptionally, the increment of resistance did not appear

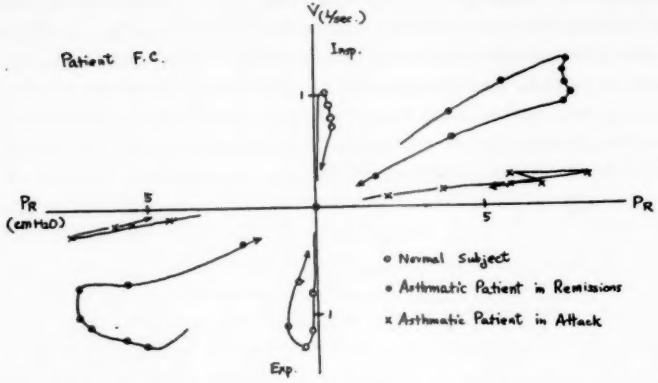

Fig. 6. Changes in pressure-flow diagram of a asthmatic patient after pilocarpin inhalation.

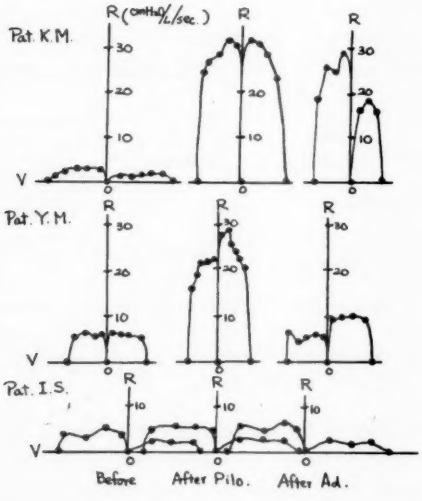

Fig. 7. Changes in volume-resistance diagrams of 3 typical cases with bronchial asthma after pilocarpin and adrenalin inhalation.

in one patient without attack by pilocarpin nebulization, as seen in Pat. I. S.

Even though 3 patients with moderate dyspnea in attack were associated with the marked pressure fluctuation in a single breathing cycle, the check valve mechanism which was reported by Dayman in his patients

with pulmonary emphysema was not observed at the beginning of expiration. Table IV shows a summary of results in mean for all phases of patients. The work for elastic resistance was 13.1 gm./L and the non-elastic work in expiration was 7.1 gm.L/L. Therefore, the ratio of these two works (elastic work/non-elastic work) is distinctly decreased relative to that in normal subjects.

TABLE IV

Mechanical Properties of the Lungs in Normal Subjects and in Asthmatic Patients during Remissions and after Pilocarpin or Adrenalin Inhalation

Subject	Phase		No. of cases	Effective compliance(L/cmH_2O)	Elastic work (gm.L/L)	Maximal resistance (cmH_2O/L/sec.)		Nonelastic work (gm.L/L)		Phase angle (°)	Power factor (Cos θ)
						Insp.	Exp.	Insp.	Exp.		
Normal			5	0.16	3.3	2.1	2.8	0.7	0.5	61	0.97
Asthmatic patients	Before		8	0.11	5.7	6.4	8.7	2.5	2.2	33	0.75
	After pilocarpin inhalation	All cases	8	0.05	13.1	30.8	38.6	7.5	7.1	14	0.92
		Dyspnea (#) cases	3	0.03	19.5	>47.0	>67.6	11.3	11.4	5	0.99
	After adrenalin inhalation		6	0.09	7.5	10.9	12.6	4.1	3.4	27	0.85

Following to the opinion of Otis,[23] one would expect that the value of the ventilation maximally effective in lowering P_ECO_2 would be lower than in the normal individual and in severe paroxyms P_ECO_2 would be elevated conversely by breathing. The power factor was nearly 1 so that all energy disappears in this system by changing to heat. Assuming intraoesophageal pressure equals -5 cm H_2O at resting expiratory level,[1] it was imagined that 3 patients who complained dyspnea moderately needed certain works of expiratory muscles in expiratory movement of lungs in addition to the potential energy stored in the lung by its elastic properties.

Table V illustrates the relationship of objective dyspnea to the work for breathing at attack, especially representing close correlation of dyspnea to the total non-elastic resistance and the pressure fluctuation in a single breathing cycle. The elastic work is considerably related to objective dyspnea except a patient S. S. with the high elastic work already prior to inhalation of pilocarpin. The fact that dyspnea appears moderately when the total non-elastic work exceeds about 20 gm.L/L and fluctuation of intraoesophageal pressure becomes more than 27–30 cm H_2O in a single

TABLE V

Dyspnea and Work for Breathing

	Grade of dyspnea	Tiffeneau's index (%)	Elastic work (gm.L/L)	Maximal resistance (cmH_2O/L/sec.)		Non-elastic work (gm.L/L)		Total Non-elastic work (gm.L/L)	Fluctuation of intraoesophageal pressure (cmH_2O)
				Insp.	Exp.	Insp.	Exp.		
Y.T.	++	28.6	25.0	77.0	102.0	13.6	14.5	28.1	34
F.K.	++	13.8	16.7	>33	>69	8.1	10.6	18.7	28
K.M.	++	12.1	16.7	31.0	31.7	12.2	9.0	21.2	27
F.C.	+	47.8	8.3	41.0	37.5	6.0	6.1	12.1	18
Y.M.	+	15.5	8.3	29.5	24.5	8.1	5.8	13.9	17
I.O.	±	51.5	8.3	11.6	14.2	5.6	4.4	10.0	15
S.S.	±	–	16.7	21.0	24.3	4.9	4.2	9.1	14
I.S.	–	69.9	5.0	2.5	5.6	1.5	2.9	4.4	11

breathing cycle would seem to confirm the following hypothesis concerning to the genesis of dyspnea, i.e. dyspnea is brought about by stimulating some presso-receptor located in the thorax.[2] Christie[12] also reported the close relationship between dyspnea and the work for breathing in cardiac patients.

The dyspnea of asthmatic patients had been considered commonly to be expiratory in quality.[1),3),4),5),6)] According to the above data, the work for breathing is closely related to dyspnea, and the non-elastic work in inspiration is same as or higher than that in expiration. Moreover, in inspiration the work for breathing is added with the work for elastic resistance, and in expiration the non-elastic work is partially performed by potential energy stored in the lung inspiratorily. For these reasons the typical dyspnea at least provocated by pilocarpin inhalation may be said to be inspiratory dyspnea rather than expiratory one. These experimental results seem to give an affirmative answer to clinical observations that asthmatic patients usually complain of inspiratory dyspnea.

It would seem to be of fundamental importance for genesis of bronchial asthma to determine separately the resistance due to air flow in bronchial canalization and the frictional resistance in the pulmonary tissue. The pressure difference between the intraalveolar pressure obtained with Vuilleumier's method and the pulmonary pressure measured by intraoesophageal balloon may occur theoretically due to the frictional resistance in the lung tissue. The experimental results of normal subjects reveal no difference between them and are consistent with the reports of Fry *et al.*[18] and Mead *et al.*[20] (Fig. 8). On the other hand, there were some varieties in results obtained from asthmatic patients at attack, demon-

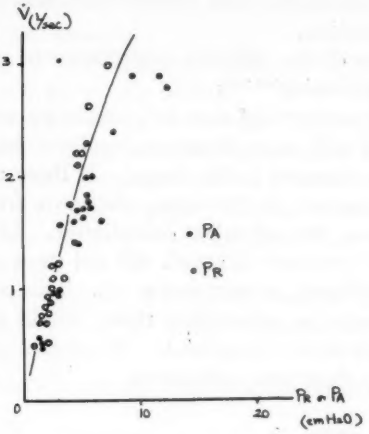

Fig. 8. Relationship between intraalveolar pressure and pulmonary pressure in each flow rate in a normal subject.
P_A: intraalveolar pressure.

strating some cases with definite differences and some case without any difference (Fig. 9). However, it is difficult to measure both the intraalveolar and pulmonary pressure during asthmatic attack mainly due to prolonged equilibration of pressures after interruption of air flow by the

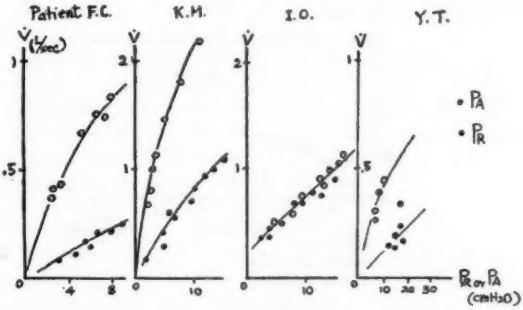

Fig. 9. Relationship between intraalveolar pressure and pulmonary pressure in each flow rate in 4 asthmatic patients during attacks.

interruption technique. In addition, Mead *et al.*[20] reported that the intraalveolar pressure obtained with Vuilleumier's method may also include the pressure difference due to frictional tissue resistance. For these reasons, it is not necessarily implied but seems to be likely that the patients F. C., K. M. & Y. T. have remarkably increased frictional resistances of the

pulmonary tissues which are more intense than that of the flow resistance in bronchial canalization.

The diminution of the effective compliance in affected lung may be caused chiefly by following[21),24)]:

I) the lung becomes stiff due to pulmonary congestion,

II) the alveoli with non-effective compliance increase due to uneven distribution of time constant in the lungs. If there are some uneven distributions of time constant in the lungs, the more frequent the breathing the smaller becomes the effective compliance. In high frequency of breathing, asthmatic patients in attack did not show any marked decrease of the effective compliance, as seen in Fig. 10. This result and the increase of frictional resistance in pulmonary tissue would seem to confirm occurance of the pulmonary congestion. However, present studies could not separate clearly these two resistances.

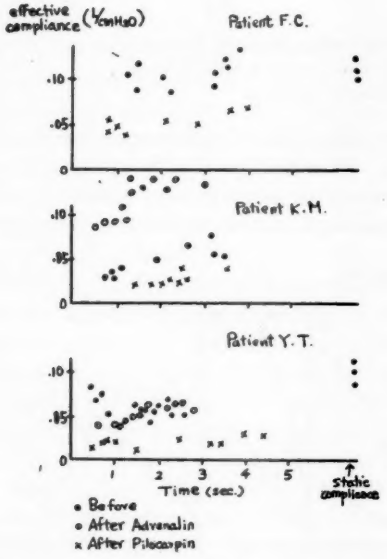

Fig. 10. Changes of the effective compliance due to changes of breathing frequency in 3 asthmatic patients. Time : time required for a single breathing cycle.

Conclusion

1. In 8 asthmatic patients, mechanical behaviors of the lung were studied during both remissions and asthmatic attack provocated with 1 :100 pilocarpin inhalation.

2. In remissions, the effective compliances of the lung in patients decrease, the maximal resistances increase considerably, and the non-elastic work is 4-fold of that in normal subjects. The mean power factor is 0.75 compared to 0.45 in the normal.

3. After the attack was induced with 1 :100 pilocarpin inhalation, VC, Tiffeneau's index and the effective compliance of the lung decreased, and the elastic work, the maximal resistance and the work for effective resistance increased distinctly except a patient without any dyspnea.

4. In attack, no check valve mechanism was observed at the beginning of expiration.

5. The objective dyspnea was closely related to the work required for total flow resistance as well as to the intrathoratic pressure fluctuation in a single breathing cycle.

6. The dyspnea was much more severe inspiratorily than expiratorily.

7. In some patients, the frictional resistance of the lung tissue increased presumably more than flow resitance in the bronchial canalization. This result seems to be explained by supposing that the pulmonary congestion occurs more intensely than the bronchial spasm does, resulting in decreased effective compliance of the lung and high frictional resistance in lung tissue.

References

1) Altschule, Physiology in diseases of the heart and lungs, Harvard University Press, Cambridge, 1948.
2) Comroe, The lung, The Year Book Publishers, Chicago.
3) Sheldon & Otis, J. Appl. Physiol., 1951, **3**, 513.
4) Sakamoto, Hagiwara & Isikawa, Shinryo (Jap.), 1953, **6**, 19.
5) Tsuji, Wesen und Behandlung des Bronchialasthma, Urban & Schwarzenberg, 1939.
6) Gottlieb, Ann. Allergy, 1955, **13**, 423.
7) Tiffeneau & Drutel, Poumon, 1955, **11**, 385.
8) Cara, C. R. de la Soc. et Biol., 1953, **147**, 1233.
9) Brown, Fry & Ebert, Am. J. Med., 1954, **17**, 438.
10) Bayliss & Robertson, Quart. J. Exp. Physiol., 1939, **29**, 27.
11) Cara, Poumon, 1955, **11**, 969.
12) Christie, Proc. Roy. Soc. Med., 1953, **46**, 381.
13) Dayman, J. Clin. Invest., 1951, **30**, 1175.
14) Dean, Am. J. Physiol., 1941, **134**, 450.
15) Dornhorst & Kelly, Lancet, 1954, No. 6806, 290.
16) Fenn, Am. J. Med., 1951, **10**, 77.
17) Fry, Stead, Ebert, Lubin & Wells, J. Lab. Clin. Med., 1952, **40**, 664.
18) Fry, Ebert, Stead & Brown, Am. J. Med., 1954, **16**, 80.
19) Mead & Whittenberger, J. Appl. Physiol., 1953, **5**, 779.
20) Mead & Whittenberger, *ibid.*, 1954, **6**, 408.
21) Mead, Lindgren & Gaensler, J. Clin. Invest., 1955, **34**, 1005.

22) Mead, McIlroy, Selverstone & Kriete, J. Appl. Physiol., 1955, **7**, 491.
23) Otis, Physiol. Rev., 1954, **34**, 449.
24) Otis, Mckerrow, Bartlett, Mead, McIlroy, Selverstone & Radford, J. Appl. Physiol., 1956, **8**, 427.
25) Stead, Fry & Ebert, S. Lab. Clin. Med., 1952, **40**, 674.